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ON THE MAINTENANCE OF STRAINS OF *SCHISTOSOMA MANSONI*, *S. HAEMATOBIUM* AND *S. MATTHEI* IN THE LABORATORY IN EGYPT, WITH SPECIAL REFERENCE TO THE USE OF GERBILS.

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THIS communication deals with the infection of small animals, including monkeys, with *Schistosoma* spp., and the maintenance of schistosome strains by the Medical Research Council unit working in Cairo in 1947–50.

Cercariae were obtained either from wild infected snails, both *Planorbis boissyi* and *Bulinus truncatus*, collected in the field by local collectors, or from snails exposed in the laboratory to miracidia or viable ova obtained from hospital patients. The average developmental period in snails in aquaria in Egypt is about 42 days. Cercariae of *S. matthei* were also obtained from *Physopsis globosa* brought by air from Southern Rhodesia.

The problem of an ideal laboratory experimental host for work on Bilharzia disease is still unsolved. Monkeys (*Rhesus* monkeys or baboons) are certainly the most suitable animals when available, but they are often difficult to obtain, expensive and delicate ; they require much cage space. Fairley (1919) has given a very full description of experimental bilharziasis in the monkey.

Various small animals have been used with partial success, e.g., white mice, gerbils, rabbits, rats, hedgehogs, hamsters, moles and guinea-pigs. Fairley (1933) used ruminants (sheep or goats) for *S. spindalis* and *S. matthei*. None of these hosts is perfectly satisfactory for laboratory work.

Mice can be fairly easily infected with *S. mansoni*, and some workers in America, Germany and London claim to have maintained a strain for years, using only viable ova from laboratory infected mice. Generally, however, the yield of viable ova from infected mice is very scanty and the yield of adult worms at autopsy is also small. Most other small animals are open to the same objection, especially regarding passage of enough viable ova in the faeces to provide miracidia. Brumpt (1928) claims to have successfully infected hedgehogs with *S. haematobium*. In most American laboratories mice and rabbits appear to be the animals of choice. Kuntz, Stirewalt and Evans (1949) were very successful

using golden hamsters and albino mice as hosts for *S. mansoni*, obtaining both adult worms and viable ova.

Krakover, Hoffman and Axtmayer (1940) used white rats successfully in Peurto Rico, but almost all the worms (*S. mansoni*) remained in the liver, and ova were not passed. Brandt and Finch (1946) describe the recovery of adult *S. mansoni* from experimentally infected rabbits. Gordon, Davey and Peaston (1934) used white rats, guinea-pigs and monkeys in Sierra Leone and found the rats refractory, while the guinea-pigs yielded adult worms, but did not pass ova, and the monkeys passed ova from 42 days after exposure. Cowper (1947) obtained viable ova from a *Rhesus* monkey in Liverpool, exposed to cercariae of *S. mansoni*, four months after exposure.

In Cairo the Medical Research Council made use of monkeys (baboons), gerbils (desert rats), white mice, rabbits, guinea-pigs and hedgehogs. In particular, gerbils were extensively used and the following paragraphs deal with work done with these animals.

EXPERIMENTS WITH GERBILS.

It must be said at once that these animals do not fulfil the requirements of an ideal laboratory animal for maintaining schistosome strains as viable ova passed in faeces are scanty and irregular, and it is therefore difficult to obtain miracidia. It was found, however, that it was extremely easy to obtain heavy infections in these animals with *S. mansoni* and thereafter to obtain a steady supply of adult males and females alive at autopsy for *in vitro* experiments, etc. Gerbils were also successfully infected with *S. matthei* and, with difficulty, with *S. haematobium*. Since the use of these animals does not seem to have been very much appreciated outside Egypt, the experiments are described in some detail.

Gerbils or desert rats belong to the genus *Gerbillus* Desmoret, Family Muridae, Subfamily Gerbillinae, and occur abundantly in the deserts of Egypt and North Africa. Anderson (1902) describes at least four species occurring in Egypt, viz.: *G. gerbillus*, *G. pyramidon*, *G. pyargus* and *G. andersoni*. Locally they are often miscalled jerboas, but the true jerboa (*Jaculus* Erxl.) is a different animal and was not used in our work. Gerbils were obtained by the Unit in numbers by purchase at a cost of two piastres (about 5d.) each from vendors who obtained them from Bedouin catchers. They were easily maintained in boxes 8 × 18 × 18 inches, with wire netting in the lids; about 6 to 12 gerbils were kept in each box and fed on dry Egyptian native bread and wet green lettuce leaves. Except in hot summer weather fluids were not necessary, green leaves providing all the moisture needed by these animals from the dry sandy deserts. Ours did not breed in captivity but deaths from natural causes were very few except during one heat-wave, when a number succumbed to temperatures of 112° to 115° F. In such extreme heat they should be kept in shade and given water or milk. Gerbils are extremely active creatures, escape easily, and give powerful bites; they are best picked up by the root of the tail with large forceps.

Exposure to cercariae.

Cercariae were collected as follows: From time to time *P. boissyi* and *B. truncatus* shed cercariae of *S. mansoni* and *S. haematobium* in the laboratory.

The snails were placed in small glass pots of filtered Nile water and then exposed to sunshine. (The city tap water was superchlorinated following a cholera outbreak and was found to be toxic to cercariae.) When examination with a hand-lens showed a good suspension of cercariae, the water was passed through gauze to remove snail faeces, etc., and 5 c.c. were pipetted up after gentle transverse stirring and placed in a circular glass dish 2 inches in diameter. A few drops of Bouin's Picro-Formol were then added—this served both to kill the cercariae and to render them easily visible against a yellow background. The number of cercariae visible in each quadrant was then counted under a binocular dissecting microscope, using a mechanical hand-counting machine. The number of cercariae in 5 c.c. of water was thus ascertained and the volume of water containing the appropriate number of cercariae was added to the vessels containing the animals. The method of semi-immersion was found to be the one of choice for infecting small animals (i.e. other than monkeys). The optimum exposure period was 30 to 45 minutes. In all cases the small animals were given a preliminary semi-immersion in clear water for up to 30 minutes, in order to remove urine and faeces before exposure to cercariae, since Adbel Azim and Watson (1948) have indicated the toxic action of urine on cercariae. A combined total immersion period of 60 to 80 minutes in water did not appear to harm the gerbils. At first each gerbil was exposed individually to a known number of cercariae in a small pot. Later it was found that equally good infections could be obtained by exposing 6 to 10 gerbils to semi-immersion in glass tanks containing a known number of cercariae, assuming an average number of cercariae per gerbil. This method is quicker, easier and avoids possible fatal injury to the gerbil when it is introduced into a small pot. Monkeys were best infected by the drip method (*v. infra*).

Autopsy methods.

To recover adult worms the liver and the gut from pylorus to rectum were removed with as much mesentery as possible, and placed in normal saline in Petri dishes. Gut and mesenteries were then teased in a Petri dish or extended on the glass stage of a dissecting microscope and examined for worms. Worms generally stand out well, the zig-zag black caeca full of changed blood pigment is clearly visible. The presence of brown bilharzial pigment was normally an indication that worms would be found. As many as three males were once observed jammed one behind the other in one mesenteric vessel. Both sexes were obtained, separate and in couples, but males always predominated. Occasionally a few worms floated out free in the saline upon immersion of the viscera without dissection. The liver was divided into four or five pieces, and if each was gently crushed between two glass slides worms could often be observed with the naked eye by holding the preparation up to the light ; subsequently they were dissected out on the microscope stage.

The tables which follow summarize the experiments carried out with gerbils and the various observations made.

Four methods of exposure were tried and compared, viz. :

(1) One gerbil semi-immersed in each of a number of small pots and a known number of cercariae added.

(2) A number of gerbils together in a tank to which a known number of cercariae were added, taking the average number of cercariae per gerbil.

TABLE I.—*Experiments with Gerbils—Results of First Twenty.*

Number of gerbils.	Parasite.	Average number of cercariae per animal and method of infection	Period of exposure in minutes.	Results.
12	*S. haematobium*	50 : semi-immersion	45	No ova passed. 3 died. All autopsied within 150 days; A total of 4 worms found 3 in one animal.
12	*S. mansoni*	50 : ,,	30	Only one ovum seen throughout. All autopsied within 156 days. All animals infected.
10	*S. haematobium*	50 : subcutaneous injection	S.C. injection	No ova passed. All autopsied within 154 days; one with 1 worm, one with 2 worms; 1 lost; others negative.
10	*S. mansoni*	50 : subcutaneous injection	S.C. injection	No ova passed. All autopsies within 152 days. No worms seen in any animals.
5	,,	60 : semi-immersion	30	Ova present in faeces after 49 days. Worms present at auptopsy within 87 days.
10	,,	400 :	40–50	No ova in faeces. Autopsies from 42 to 143 days. Worms present in all except one.
10	,,	400 : ,,	30	Six out of 10 showed numerous worms from 46th day; others lost.
8	,,	300 : ,,	40–45	Ova present in faeces. All showed worms at autopsy; last 3 yielded 25, 45 and 21 worms.
9	,,	300 : ,,	40	No ova in faeces. All infected with worms after 129 days. Infections very heavy; 48, 88, 36 and 94 worms at various autopsies.
2	,,	180 : ,,	30	27 and 18 worms at autopsy after 41 days, including immature Schistosomulae in young couples.
12	,,	200 : ,,	30	Faeces showed ova. Two showed worms at autopsy after 99 days; others lost.
12	,,	400 : ,,	30	Faeces showed no ova. All autopsies showed worms after 93 days.
12	,,	600 : ,,	30	Two showed worms at autopsy; others lost.
6	,,	100 : ,,	35	Three showed worms at autopsy after 98 days.
10	,,	80 : ,,	30–40	Worms found in all at autopsy.
5	,,	200 : ,,	30–35	Up to 44 adult worms at autopsy after 92 days.
5	,,	100–200 : semi-immersion	35	Apparently one spontaneous cure. A few worms present at other autopsies after 151 days.
5	*S. Mathei*, ex-Rhodesian *Physopsis*	90 : semi-immersion	30	11 immature worms with one coupled pair after 35 days. Worms present at autopsy after 44 days.
5	Do.	100 in 2 c.c. water by intraperitoneal injection	I.P.	One autopsy negative after 34 days. Adult couples recovered at autopsy in two after 105 days.

(3) Subcutaneous injection of a known number of cercariae with a 10 c.c. record syringe.

(4) Intraperitoneal injection of a known number of cercariae in water (*S. matthei*).

The second method was eventually the one of choice ; the third was abandoned as greatly inferior to the other though it is recommended by Kikuth in Germany. In this, our findings agree with those of Abdel Azim and Watson (1948), who compared the results of various methods of infecting mice with *S. mansoni* and concluded that semi-immersion was the most satisfactory method.

Observations and conclusions.

It will be seen from the above table that of the three various methods of infection tried by us, semi-immersion is the most efficient and provides a reliable yield of adult *S. mansoni* worms. While gerbils were also successfully infected with *S. haematobium* the yield of adult worms was very scanty. Subcutaneous injection was early abandoned as unsatisfactory ; intraperitoneal injection, as used successfully by workers in Rhodesia, was apparently successful in infecting guinea-pigs with *S. matthei*, but as it was combined with semi-immersion to assure infection, its exact value was unproven. Gerbils were also infected with *S. matthei* both by the intraperitoneal route and by semi-immersion and yielded adult worms.

The following observations were made on the infection of gerbils with *S. mansoni* :

(1) *Proportion of male to female adult* S. mansoni *in gerbils.*—Out of a sample of 30 gerbils exposed to numbers of *S. mansoni* cercariae varying from 80 to 600, the following findings were made at autopsies carried out 41 to 172 days after exposure.

In 30 autopsies 690 males and 256 females were recovered. Of these females 235 were coupled and 21 free. The proportion of males to females was 2·7 to 1. All-male infections were not encountered, the cercariae always coming from a number of different individual snails (Fig. 1).

(2) *Relationship between number of adult* S. mansoni *recovered from liver and from mesentery* (Fig. 2).—There was a definite preponderance of adult worms

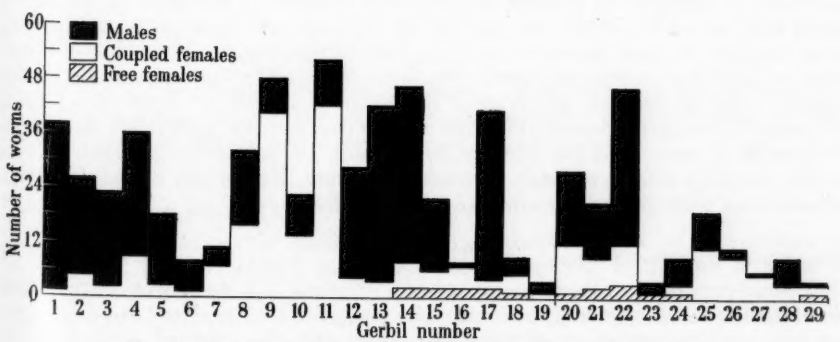

FIG. 1.—Proportion of male to female adult *S. mansoni* in gerbils.

recovered from the mesenteries over those recovered from the liver. This is illustrated by Fig. 2. It will be noted that two of the gerbils show an abnormal number of worms in the mesentery (No. 10 and 12). At this point the appearance of the graph is atypical. Autopsies on 30 gerbils exposed to from 80 to 600 cercariae were carried out from 41 to 172 days after exposure and showed that numbers of worms varying from 1 to 32 were recovered from the liver and varying from 0 to 94 from the mesentery. In 11 cases there were more worms in the liver than in the mesentery; in 18 more in the mesentery than in the liver. In 30 autopsies altogether there were 351 worms in the liver to 599 in the mesenteries, or a proportion of 1·7 in the mesentery to 1·0 in the liver.

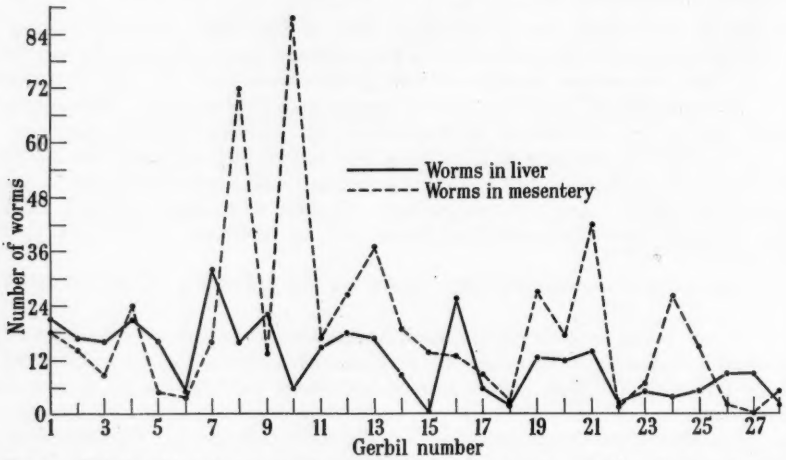

FIG. 2.—Relationship between number of adult *S. mansoni* recovered from liver and from mesentery.

(3) *Correlation between numbers of cercariae at exposure and number of worms recovered.* (See Fig. 3 and Table II).—Table II shows the number of worms recovered at autopsy in relation to the mean number of cercariae to which the gerbil was exposed. While the number of cercariae was necessarily an approximate average, so that figures are of only limited significance, it would seem that there was no exact numerical correlation to be observed in this series of 37. It also appears that no marked increase in the yield of worms is obtained by using numbers of cercariae greater than 200 to 300 average per gerbil. If more than 300 cercariae were used the yield of worms was not appreciably larger. These results are very similar to those obtained by Kuntz, Evans and Stirewalt (1949), who worked with golden hamsters and albino mice.

Recovery of immature worms. (See Table II).

Immature worms or Schistosomulae, of both sexes of *S. mansoni* were recovered in both liver and mesentery at autopsies on gerbils carried out 41 days after exposure to 360 cercariae. Since these autopsies also yielded some couples, it follows that coupling of males and females must have commenced not later

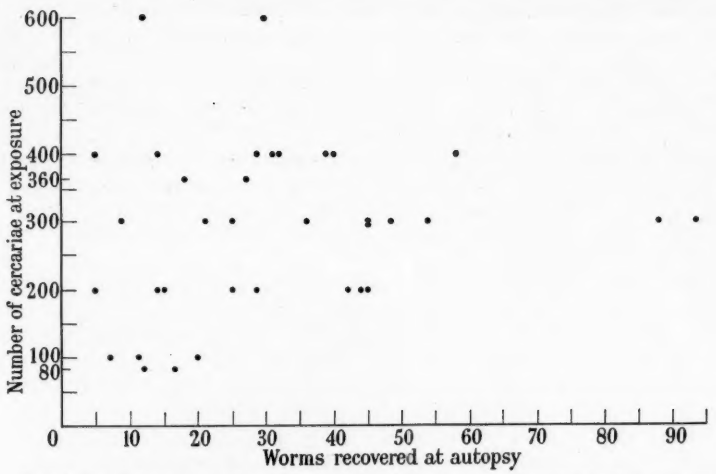

FIG. 3.—Correlation between numbers of cercariae at exposure, and number of worms recovered.

TABLE II.—*Relation between Number of Cercariae and Number of Worms Recovered.*

S. mansoni ex-Egyptian *Planorbis* in 35 Gerbils.

Approximate number of cercariae.	Number of worms recovered at autopsy.
80	16, 12
100	20, 11, 7
200	28, 15, 45, 14, 5, 44, 25, 42
300	25, 45, 21, 9, 48, 88, 36, 94, 45, 54
360	27, 18
400	14, 39, 31, 32, 40, 29, 58, 5
600	12, 30

than 41 days after infection or possibly earlier. In the case of *S. matthei* in experimental guinea-pigs, uncoupled immature worms were recovered after 32 days from liver and mesentery and a coupled pair after 35 days.

Passage of eggs in faeces.

The occurrence of the characteristic lateral-spined ova of *S. mansoni* in the faeces of infected gerbils was scanty and irregular, Search for ova has no diagnostic value as gerbils with negative faeces may carry a heavy worm load. The number of viable ova obtained was not sufficient to provide miracidia to maintain a strain. Rectal scrapings were not carried out but might be of value in this connection. In this respect gerbils are quite inferior to monkeys.

THE PATHOLOGY OF *S. mansoni* INFECTION IN GERBILS.

Paraffin sections of liver, stomach, intestine and spleen were studied. We are indebted to Dr. M. Hashem, of the Pathology Department, Faculty of Medicine, Cairo, for the report which follows.

At autopsy the liver nearly always appeared macroscopically normal, even with heavy worm loads. In almost all cases the intestine showed numerous yellowish-white, semi-translucent, subserous pseudotubercles. Heavy worm loads did not appear to prevent gerbils remaining active and healthy. Infections in mice are much lighter.

Intestine.—The bilharzial lesions in general are more extensive in the large than in the small intestine. Bilharzial granulation tissue is found involving mostly the peritoneal and muscular coats and to a less extent the mucous membrane which shows extensive catarrhal inflammation (Fig. 4). The lesions are found either as aggregations of bilharzial pseudotubercles or as diffuse miliary granulation tissue. In the former, a degenerated and collapsed ovum is found in the centre surrounded by endothelioid cells with some eosinophils, plasma cells and lymphocytes. Some of the histiocytes contain brownish, bilharzial pigment and at the periphery show a fibroblastic reaction. The reaction is so extensive that in some parts the whole muscular coat is almost destroyed. The lymphoid follicles are also infiltrated by scattered bilharzia ova mostly with no reaction around them. The subperitoneal adipose tissue is also in some parts infiltrated by ova with the usual reaction. Where the ova affect the mucous membrane the lesions are usually in the deeper layers of the mucosa exciting granulation tissue around them, and replacing portions of mucous membrane *in situ*. In the mouse sections a nematode worm which could not be identified was found in the lumen (*Syphacia* sp.). Pancreatic tissue was found related to the wall of the intestine and showed multiple bilharzial pseudotubercles in the interstitial tissue producing local destruction in the pancreatic tissue.

Liver.—There is congestion and cloudy swelling of the parenchymatous tissue. The Küpffer cells are hypertrophied, proliferated and contain dark brown bilharzial pigment. Some of the small portal tracts are infiltrated with bilharzia ova surrounded by bilharzial reaction and some similar nodules are found without ova amongst them. Apart from the bilharzial nodules the portal tracts in places are infiltrated with chronic inflammatory cells, particularly histiocytes and plasma cells and a few eosinophils, amongst which are found some bilharzia ova and much dark brown bilharzial pigment. There is evidence of fibroblastic reaction. These tracts showed a tendency to partial enclosure of original hepatic lobules or groups of lobules ; a picture which can be considered as an early stage of fine peripheral bilharzial fibrosis (Fig. 5).

Some of the larger portal venules contain degenerated bilharzia ova. A collapsed ovum was found in a small venule which from its position may be considered a central vein. In one section, a section of a female bilharzia worm was found in a small portal tributary producing no reaction.

Spleen.—In some parts there is evidence of reticulo-endothelial hyperplasia with some foreign-body giant cells. The pulp is densely packed with red blood corpuscles. There are numerous dark brown granules of bilharzia pigment. The malpighian bodies are composed mainly of lymphocytes with only slight

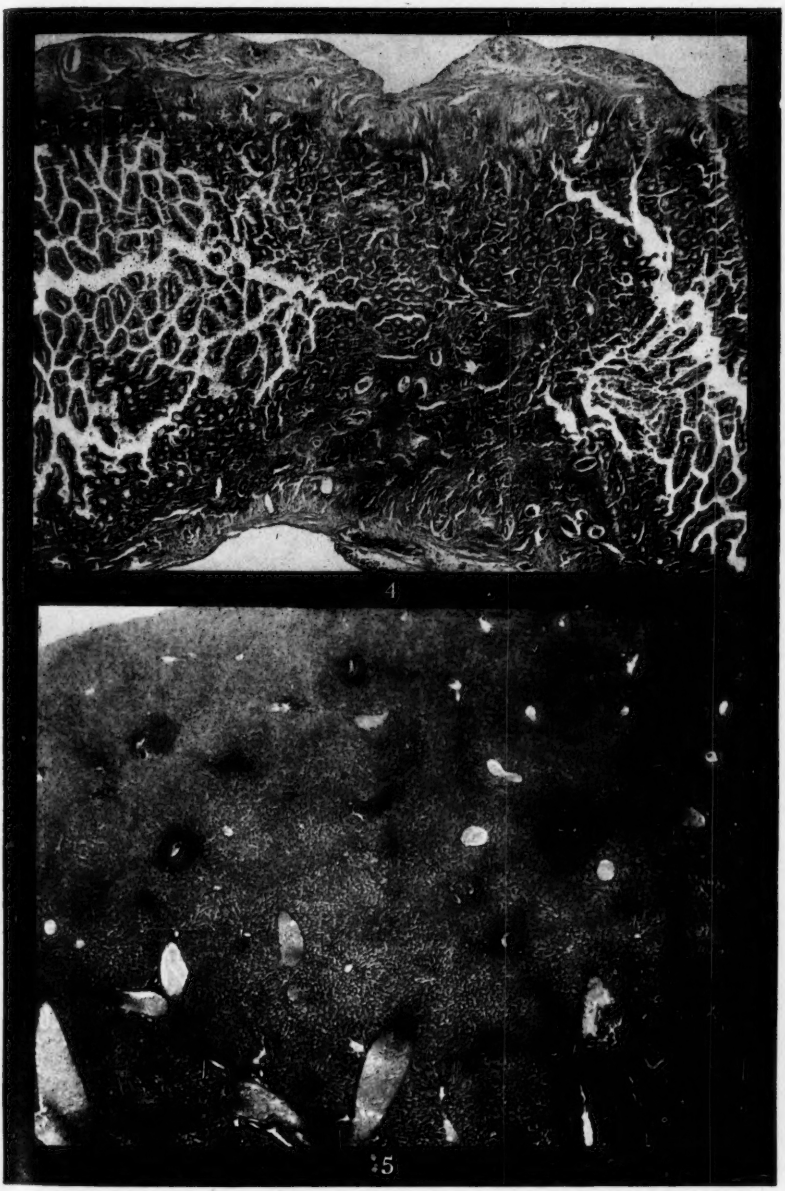

FIG. 4.—Intestine of gerbil infected with *S. mansoni*, longitudinal section, showing bilharzial pseudoturbercles and fibrosis round ova in subserous muscular and submucous layers.
FIG. 5.—Liver of mouse. Pseudotubercles surrounding ova of *S. mansoni* and very early cirrhosis.

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evidence of reticulo-endothelial hyperplasia. Many clumps of bilharzial pigment are seen among them. No bilharzia ova could be found in the spleen.

EXPERIMENTS WITH MICE.

(Table III.)

White mice have been used for the maintenance of strains of *S. mansoni* in Germany, England and America. In Cairo we found them to have the following disadvantages :

(1) They died easily and in large numbers.
(2) The yield of adult worms was much smaller than in gerbils.
(3) Viable eggs, though passed in the faeces, were few and irregular.
(4) They were considerably more expensive to buy than gerbils.

Accordingly mice were not used as experimental animals for routine work, gerbils being employed instead.

Newborn mice were exposed in watch-glasses to cercariae of *S. mansoni* and *S. matthei*. Table III shows the details of these experiments. Sections of entire mice fixed in Bouin's picro-formol, were cut and mounted, and penetrating cercariae observed in the sub-epidermal layers as figured by Leiper (1915).

TABLE III.—*Experiments with Mice.*

Number of mice.	Parasite.	Average number of cercariae per animal and method of infection.	Period of exposure in minutes.	Results.
4 adults	*S. haematobium*	100 : semi-immersion	30–45	Last autopsy after 122 days No worms, ova or segments found.
12 ,,	*S. mansoni*	100 : ,,	30	Ova found in faeces after 59 days. 3 died. 8 out of 9 contained worms at autopsy ; not more than 6 worms in any one mouse. Stoll egg-count 50–60 eggs/c.c. or 600 eggs/g. faeces.
12 ,,	,,	60–80 semi-immersion	50	None contained worms at autopsy after 141 days.
8 adults	,,	70–80 : semi-immersion	40	All died soon after infection.
5 ,,	*S. haematobium*	110 : semi-immersion	30	All but one died soon after infection. One autopsy negative after 88 days.
5 newborn	,,	130, 120 and 140 : semi-immersion	15	One killed at once, 2 after 3 days. Cercariae seen in sub-epidermal layers.
1 newborn	*S. matthei*	60 : ,,	30	Killed after 30 minutes; cercariae observed in sub-epidermal layers.
1 ,,	,,	100 : semi-immersion (shed tails observed after exposure)	30	Killed after 25 minutes ; cercariae observed in sub-epidermal layers.

An attempt to infect mice with *S. haematobium* was unsuccessful. Apart from the newborn mice described above, mice were not exposed to *S. matthei*.

A Stoll egg-count carried out on one mouse infected with *S. matthei* and passing ova in the faeces gave a count of 50 to 60 eggs per c.c., or approximately 600 eggs per g. of faeces.

EXPERIMENTS WITH GUINEA-PIGS, RABBITS AND HEDGEHOGS.

(Table IV.)

Guinea-pigs.

These were infected with *S. matthei* ex-Rhodesian *Physopsis*. They were given 100 to 120 cercariae in water (not sterile) by intraperitoneal injection followed by 600 to 750 cercariae by the semi-immersion method. Only one death, possibly due to peritonitis, occurred, following injection. Guinea-pigs are considered the best host for *S. matthei* by the Rhodesian workers. In our own animals immature worms were recovered at autopsy from 32 days onwards. Adult *S. matthei* were recovered after 63 days. Two guinea-pigs sacrificed about 6 months after injection had either undergone spontaneous cure or failed to become infected ; probably the former, since the others were heavily infected.

Rabbits.

As shown in Table IV, 3 rabbits were exposed to *S. mansoni* and in each case a different method of infection was used ; 400 to 800 cercariae were given. In no case were any adult worms recovered at autopsy. In America rabbits have been successfully infected with *S. mansoni* (Brandt and Finch, 1946).

Hedgehogs.

Brumpt (1928) successfully infected hedgehogs with *S. haematobium*. He notes that eggs and worms were present in the gut and pancreas but not in the bladder. Out of the first 6 hedgehogs which we exposed to *S. haematobium* cercariae ex-Egyptian *Bulinus* (average 500 to 600 cercariae each) by the semi-immersion method, one subsequently passed a few terminal-spined ova in the faeces after 80 days. No ova were seen in subsequent examinations and it appeared that the infection underwent spontaneous cure. In two others the faeces remained negative for eggs. No worms were found at three autopsies. Two more, exposed to 1000 cercariae of *S. haematobium* for 30 minutes died soon after, perhaps from hyperinfection.

Two hedgehogs were exposed to 4500 *S. matthei* cercariae ex-Rhodesian *Physopsis* by semi-immersion for 35 minutes. One of these escaped and the second died after 58 days, and at autopsy yielded immature worms, mostly from the liver, including one young coupled pair. These were almost certainly *S. matthei*, but as the *Physopsis* were said to have been exposed to miracidia of both *S. matthei* and *S. haematobium* in Rhodesia it might conceivably have been another *S. haematobium* infection.

Monkeys (see Table V).

The monkeys exposed to *S. mansoni*, *S. matthei* and *S. haematobium* were young or medium-sized baboons (*Papio* sp.) from Eritrea.

The experimental work done on these is to be described in detail later. Table V shows the results in the case of 6 monkeys exposed to the three schistosome species.

TABLE IV.—*Experiments with Rabbits, Guinea-pigs and Hedgehogs.*

Number of animals.	Parasite.	Average number of cercariae per animal and method of infection.	Period of exposure in minutes.	Results.
1 rabbit	*S. mansoni*	400 : subcutaneous injection	S.C.I.	No worms found at autopsy after 85 days.
1 „	„	400 : to shaved skin	20	No worms found at autopsy after 70 days.
1 „	„	800 : semi-immersion	30	No worms found at autopsy after 72 days.
6 hedgehogs	*S. haematobium*	600 : „	45	Only one passed ova after 80 days.
3 „	„	1000 : „	30	All died, possibly from over-infection.
2 „	*S. matthei*	4500 : „	45	One lost; 1 died after 58 days. Immature worms present.
5 guinea-pigs	„	100 : intraperitoneal injection; 750 : semi-immersion	I.P. 40	One contained immature worms after 32 days. No ova in faeces after 42 days. One spontaneous cure. Two died early.
5 „	„	120 : intraperitoneal injection 600 : semi-immersion	I.P. 40	Two died after 63 days; heavy infection of liver and mesentery with adult worms, mostly in mesentery. No ova in faeces of any after 52 days. One autopsy showed no worms after 133 days. ? Spontaneous cure.
3 „	„	300 : „	30	Two died early and showed no worms. One died after 36 days; contained 6 worms.
2 „	„	600 : „	30	Adult worms recovered.

Examination of the monkeys' blood did not yield any blood parasites. Some of the baboons were naturally infected with *Enterobius* sp. and *Trichuris* sp.

The methods employed were as follows :

(1) *Semi-immersion.*—This took place in a zinc tray at the bottom of a small cage, following a previous semi-immersion in clean water to remove faeces and urine. The monkey was " paddled " in about 3 inches of water, generally at a temperature of about 32° C., and the cercariae were then added through the base of the cage or splashed into the monkey's face. Exposure was for about one hour and the number of cercariae used varied from 2000 to 5000.

(2) *Drip method.*—Cercariae were dripped with a pipette on to the belly, nose, mouth and ears of an anaesthetized monkey, or a monkey held or tied down. On one occasion this was combined with standing a tube containing cercariae on the skin of the belly. Light ether anaesthesia was found to be the safest method on the whole, and did the monkey no harm. A small dog muzzle and a pad of cotton wool make a satisfactory anaesthetic mask. Chloroform was tried, but abandoned as too dangerous.

(3) *Modified semi-immersion.*—In this method the fore-part of the trunk only was immersed, thus obviating the need for a preliminary immersion to remove faeces and urine.

By the above methods baboons were successfully infected with *S. matthei* ex-Rhodesian *Physopsis*, and *S. mansoni* ex-Egyptian *Planorbis*; ova were passed and miracidia obtained from about 60 to 70 days after infection. Successful infections were obtained with as few as 1600 cercariae and exposure periods as low as 10 minutes. On the other hand, two baboons exposed to *S. haematobium* ex-Egyptian *Bulinus* had not begun to pass ova after 130 to 150 days. This work is continuing.

TABLE V.—*Exposure of Six Baboons to* S. mansoni, S. haematobium and S. matthei.

Number of baboons.	Parasite.	Average number of cercariae per animal and method of infection.	Period of exposure in minutes.	Results.
1 young	*S. mansoni*	1st dose: 2000–3000, drip method; 2nd dose: 2600, drip method	20–30	Ova found in faeces after 66 days without centrifugation. *Enterobius* and *Trichuris* ova present.
1 medium-sized	*S. matthei*	3000: semi-immersion	60	Ova seen after 67 days in sieved and centrifuged stool.
	S. mansoni	2500: drip method	25	A few ova present after 28 days.
1 medium-sized	*S. matthei*	4500: semi-immersion (water temperature 32° C.)	60	Ova present in sieved and centrifuged faeces after 67 days.
1 medium-sized	*S. haematobium*	1700 freshly shed 3900 24 hours old: semi-immersion. Water temperature 30° C.	40	No ova found by HCl-ether concentration method after 150 days. *Enterobius* and *Ankylostoma* ova present.
1 medium-sized	„	1600: semi-immersion 300: drip method	40 20	No ova found by HCl-ether concentration method after 130 days.
1 medium-sized	*S. mansoni*	1600: drip method 1100: „	10 15	Ova present in faeces after 109 days. *Enterobius* ova present.

COMMENTARY AND SUMMARY.

When they can be obtained and maintained monkeys are easily the best hosts and seem to be the only ones which produce a regular supply of viable ova from which miracidia can be obtained. Baboons were easily infected with *S. mansoni* and *S. matthei* by both the semi-immersion and drip methods.

Gerbils are very easily infected by the semi-immersion method with *S. mansoni*. They provide a reliable yield of adult male and female worms, but ova in the faeces are scanty and irregular. The pathological changes observed in gerbils are described. Very early stages of hepatic cirrhosis were observed in gerbils infected with *S. mansoni*. There is some evidence that occasional spontaneous cure occurs, leaving a seriously damaged liver and intestine in an animal in which adult worms are few or absent at autopsy. About 200 to 300 cercariae (average per gerbil) will produce a maximum yield of worms and there is no exact correlation between the number of cercariae at exposure and the number of adult worms recovered at autopsy. The graphs show the ratio of male to female worms, coupled and free, as 2·7:1, and indicate a slight preponderance of worms in the mesentery as compared to the liver.

In addition to *S. mansoni* a few gerbils were infected successfully with *S. matthei* (Rhodesian strain) and also with *S. haematobium*, but the yield of adult *S. haematobium* was very small.

White mice were successfully infected with *S. mansoni* by semi-immersion but they were considered less suitable than gerbils for routine work for reasons detailed above. Penetrating cercariae of *S. mansoni* and *S. matthei* were demonstrated in sections of newborn mice after exposure in watch-glasses for about half an hour. An attempt to infect mice with *S. haematobium* failed, as did also an attempt to infect rabbits with *S. mansoni* by three different methods.

In an attempt to repeat the work of Brumpt (1928), one hedgehog was successfully infected with *S. haematobium* to the extent that characteristic ova were observed in its faeces on one occasion, but the infection seemed to die out spontaneously. A hedgehog was also successfully infected with *S. matthei*. It was not considered, however, that hedgehogs were suitable for large-scale routine work.

Guinea-pigs were infected in Cairo with a Rhodesian strain of *S. matthei* and numerous adult worms were obtained but two of the guinea-pigs appeared to undergo spontaneous cure in about 6 months.

The work described above was carried out in the laboratories of the Bilharzia Snail Destruction Section of the Ministry of Public Health in Cairo. Thanks are due to Sir A. T. Shousha Pasha for his interest and support, to Dr. C. H. Barlow and the technical staff of the Section, particularly Farahat Ali el Wakkad, and to Mr. E. C. England for their valuable help. We are further indebted to Professor N. Hamilton Fairley, C.B.E., F.R.S., Professor J. C. Buckley and Major-General Sir John Taylor, C.I.E., of the London School of Hygiene and Tropical Medicine, and the Medical Research Council for their support and advice from England.

REFERENCES.

Abdel Azim, M., and Watson, J.—(1948) *J. Egypt. publ. Hlth. Ass.*, **24**, 121.

Anderson, J.—(1902) 'Mammals of Egypt.' London.

Brandt, J. L., and Finch, E. P.—(1946) *Proc. soc. exp. biol., N.Y.*, **61**, 22.

Brumpt, E.—(1928) *Ann. Parasit. hum. comp.*, **6**, 440.

Cowper, S. G.—(1947) *Ann. trop. Med. Parasit.*, **41**, 173.

Fairley, N. H.—(1919) *J. Path. Bact.*, **23**, 289.—(1933) *J. Helminth.*, **11**, 181.—(1933) *Ibid.*, **11**, 181.

Idem and Jasudasin, F.—(1926) *Indian J. med. Res.*, **14**, 701.

Gordon, R. M., Davey, T. H., and Peaston, H.—(1934) *Ann. trop. Med. Parasit.*, **28**, 323.

Krakower, C., Hoffman, W. A., and Axtmayer, J. H.—(1940) *Puerto Rico J. publ. Health trop. Med.*, **16**, 269.

Kuntz, R., Stirewalt, M. A., and Evans, A. S.—(1949) Naval Medical Research Institute, National Naval Medical Centre, Bethesda, Maryland, Rep. No. 21, Project NM 005, 005 (X-535), February 8.

Idem, Evans, A. S., and Stirewalt, M. A.—(1949) *Ibid.*, Rep. No. 23, Project 005, 004 (X-535).

Leiper, R. T.—(1915) *J. Roy. Army Med. Corps*, **25**, 1.

THE EFFECT OF DIET ON OESTROGEN INACTIVATION BY THE LIVER.

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OVARIECTOMIZED female rats with oestrogen pellets implanted into the spleen are known to be capable of inactivating the oestrogen and maintaining the anoestrus state (Biskind and Mark, 1939; Segaloff, 1945). Gross dietetic liver damage induced in such rats results in impairment of the liver function of inactivating oestrogens and in a return to the oestrus state. This fact was demonstrated by Unna, Singher, Kensler, Taylor and Rhoads (1944), by Shipley and György (1944) and by György (1945). Using low protein diets these workers obtained a combination of fatty infiltration, fibrosis and necrosis of the liver.

Since Himsworth and Glynn (1944) have demonstrated that dietetic massive hepatic necrosis and diffuse fibrosis of the liver are separate entities produced by different deficiencies, it seemed of interest to see if oestrogen inactivation was similarly affected by the different pathological lesions and further to determine at what stage in the development of each lesion failure to inactivate first appeared.

MATERIAL AND METHODS.

Sixty adult female virgin Wistar rats, about 200 g. body weight, were ovariectomized. Subcutaneous injections of 2 μg. stilboestrol were used to test their ability to react to oestrogens. Fifteen to twenty-one days later, one pellet containing 5 mg. α-oestradiol was implanted into the spleen of 48 of these animals and into the muscles of 5 of them. Six ovariectomized rats were left without any implant.

These rats were kept on the normal stock diet, as follows:

Ground wheat	19·2	Meat and bone meal	9·5
Fine bran	19·2	Dried skimmed milk	7·0
Sussex oats	19·2	Dried yeast	1·3
Ground maize	9·5	Salt	0·4
Ground barley	9·5	Cod liver oil	0·4
Fish meal	4·8		
		Total	100·0

(Supplied by the N.E. Agricultural Co-operative Society, Ltd., Aberdeen.)

Vaginal smears were examined twice a week during the whole experimental period, using Long and Evans' technique (1922). Twenty days after the implantation, the rats which had shown more or less permanent oestrus since the implantation were discarded, adhesions around the spleen probably allowing direct access of the oestrogens to the systemic circulation. Only those rats which had

* Working with a grant from the French Direction generale des Relations culturelles.

shown a permanent anoestrus state for more than a week were used for the dietetic experiment.

The composition of the diets and the amount of food allowed to each rat are shown in Table I. Diet 1 (Table I) was deficient in sulphur containing amino-acids and tocopherol, yeast being the only source of protein. This diet had been used in this laboratory to produce massive liver necrosis in rats (Glynn and Himsworth, 1944). The same diet when supplemented with L-cystine (50 mg./day) (Glynn, Himsworth and Neuberger, 1945) or α-tocopherol (15–20 mg. twice a week) did not produce liver necrosis (Himsworth and Lindan, 1949). Diets 2 and 3 (Table I) were deficient in lipotropic factors and were used to produce fatty infiltration (Glynn, Himsworth and Lindan, 1948). Choline chloride as a supplement to these diets was proved to have a lipotropic effect and to prevent or to cure the fatty infiltration.

These diets were fed to different groups of rats, as shown in Table II. Five

TABLE I.—*Composition of Diets.*

	Diet 1 (yeast diet).	Diet 2 (starch diet).	Diet 3 (lard diet).
Casein (g. per cent)	0	8	12
L-cystine (g. per cent)	0	0·6	0·4
Dry baker's yeast (g. per cent)	18	0	0
Corn starch (g. per cent)	73	82·4	49
Lard (g. per cent)	0	0	30
Salt mixture (g. per cent)	3	3	2·6
Olive oil (per cent by volume)	5	5	5
Cod liver oil (per cent by volume)	1	1	1
Vitamin B complex* solution (ml./rat/day)	0·5	0·5	0·5
Allowance g./rat/day	8	10	7

* Thiamine hydrochloride . . . . . 20 μg.
Riboflavine . . . . . 25 „
Pyridoxine . . . . . 20 „
Calcium pentothenate . . . . . 100 „
Per 0·5 ml. solution.

TABLE II.—*Arrangement of Rats and Diets.*

Experiment.	Number of diet (see Table I).	Number of rats.	Ovariectomy.	Oestradiol implantation. Into the spleen.	Oestradiol implantation. Into the muscle.
Sulphur amino-acid and tocopherol deficiency	1	10	+	+	—
Lipotrophic factors deficiency	2	8	+	+	—
	3	10	+	+	—
	3	5	+	—	+
	3	6	+	—	—
	3	6	—	—	—
Control	Stock diet	5	+	+	—

ovariectomized rats with oestradiol implants in the spleen were maintained on the stock diet. Six normal rats, 6 ovariectomized and 33 ovariectomized rats with oestradiol implants were divided into several groups and given different diets. When rats given these diets showed oestrus, supplements of l-cystine (50 mg./day), α-tocopherol (25 mg./day) or choline chloride (10 mg./day), according to the factor in which the diet was deficient, were added in order to restore the normal condition of the liver.

The urine of some rats was tested for oestrogen content by the biological test of Emmens (1950).

Chemical estimations of total liver nitrogen (micro-Kjeldahl method) and fat (Leathes and Raper, 1925) were performed. Haematoxylin and eosin were used for routine histological examinations.

RESULTS.

Oestrus and Diet.

The effects of the diets on oestrus are summarized in Fig. 1 and 2.

1. *Low sulphur amino-acids, low tocopherol diet.* (Fig. 1).

Nine of 10 rats fed the yeast diet showed permanent oestrus within 11 to 37 days (average 20 days).

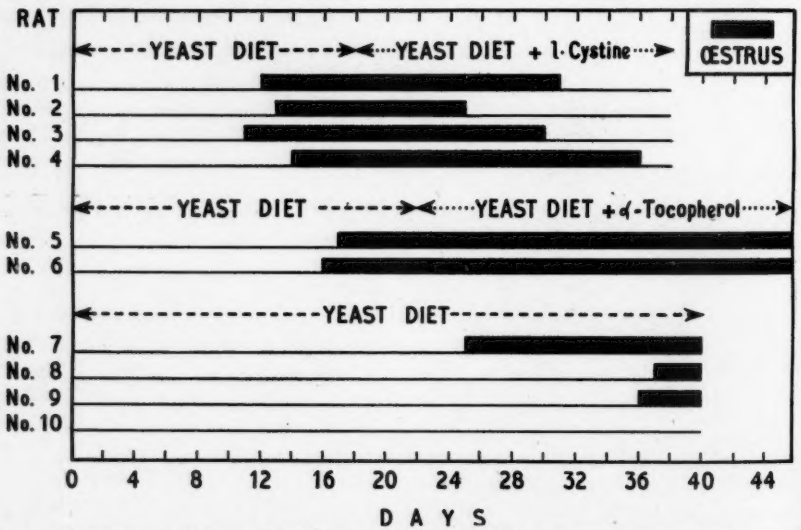

FIG. 1.—Effect of yeast diet and of cystine or tocopherol supplements upon oestrus state in ovariectomized rats with oestradiol implants in the spleen.

All 4 rats whose diet was supplemented with 50 mg. l-cystine a rat a day, after the occurrence of oestrus, returned to an anoestrus state within 7 to 18 days (average 12·5 days).

The 2 tocopherol-supplemented rats failed to return to the anoestrus state within 24 days.

2. *Alipotropic diets.* (Fig. 2.)

(*a*) All 18 rats with oestradiol implanted in the spleen and given these alipotropic diets showed permanent oestrus within 7 to 37 days, depending upon the diet used. Choline supplement resulted in return to the anoestrus state in 10 of 11 animals. By adding or withdrawing choline, it was possible to obtain a corresponding alternation of oestrus and anoestrus states. Oestrus was induced in a shorter time by Diet 3 (7 days) than by Diet 2 (approximately 25 days). Choline supplement was needed for longer with Diet 3 than with Diet 2 before oestrus disappeared.

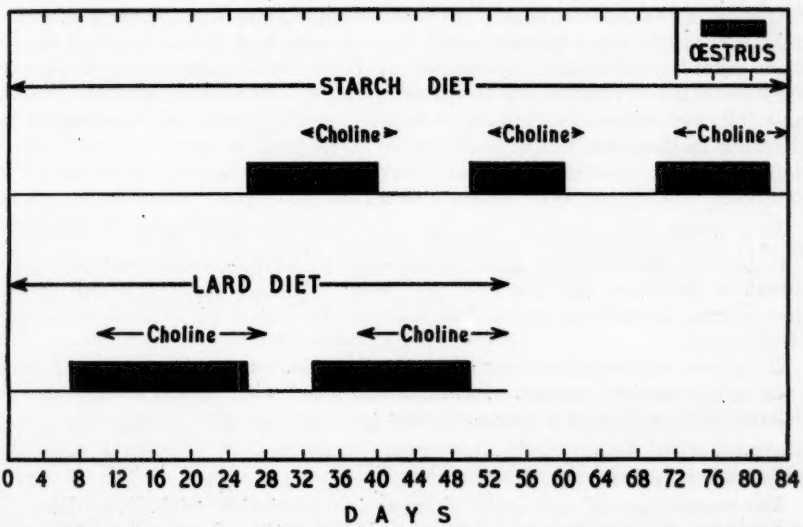

FIG. 2.—Effect of alipotropic diets and of choline supplement upon oestrus in ovariectomized rats with or without oestradiol implants in the spleen.

(*b*) Six ovariectomized rats with no oestrogen implant and given the alipotropic Diet 3, showed typical oestrus vaginal smears within 7 to 12 days. The biological assay for oestrogens of their urinary extract on spayed mice was positive. Similar assay on the urine of ovariectomized rats on stock diet was negative.

(*c*) Six non-ovariectomized, non-implanted rats given the alipotropic Diet 3 maintained normal oestrus cycles during the whole experimental period.

(*d*) Five ovariectomized rats with intramuscular implants and given the alipotropic diets remained in oestrus during the whole experimental period.

3. *Normal stock diet.*

None of the 5 ovariectomized rats with splenic implants maintained on the normal stock diet showed any oestrus throughout the whole experimental period.

Histological Findings in the Liver.

(a) *Low sulphur amino-acid, low vitamin E diet.*

All experiments were carried out before massive hepatic necrosis developed. It was noted that in some areas liver cells were swollen, vacuolated and stained poorly; but no fibrosis, necrosis or haemorrhage was present. Such swelling appeared less in the animals whose diet was supplemented with cystine.

(b) *Diet producing fatty infiltration.*

In rats killed as soon as oestrus occurred the only lesion found was fatty infiltration of the parenchyma. No trace of diffuse hepatic fibrosis was seen and would not have been expected until the animals had taken the diet for much longer periods. Further, the degree of fatty infiltration required to permit the establishing of oestrus was surprisingly light. The disappearance of oestrus which followed the administration of choline coincided with the lessening of fatty nfiltration in the liver.

Chemical Findings.

As a whole, the chemical findings paralleled the histological picture.

1. In rats fed the diet deficient in sulphur amino-acids the total nitrogen content of the liver was low (2·1 per cent). Though higher, it still remained below normal in animals whose diet was supplemented by cystine or tocopherol (2·6 per cent).

2. In rats fed the alipotropic diets, the liver fat content was about 12 per cent on the appearance of oestrus. In animals in whom choline had abolished oestrus, the liver fat was about 4 per cent.

DISCUSSION.

The occurrence of endocrine changes in association with liver disease has attracted much interest in recent years. Many authors have referred to gynecomastia, testicular atrophy, loss of axillary hair, vascular arterial spiders and palmar erythema occurring in the course of cirrhosis of the liver. The urinary excretion of oestrogenic substances during liver disease has been found to be increased. These findings have been interpreted as arising from failure of the cirrhotic liver to inactivate circulating oestrogens (Biskind and Biskind, 1946).

In 1947, however, Klatskin, Salter and Humm described the association of gynecomastia, testicular atrophy, cutaneous spider naevi and palmar erythema in the absence of evident liver disease. The subjects were American soldiers who had suffered from malnutrition as prisoners of war in Japan. Only one half of those affected showed either hepatomegaly or impaired liver function. In the other half the liver appeared normal, the feminizing changes being the only clinical abnormalities. Nevertheless, the authors suggested the possibility of liver dysfunction as a cause of the clinical signs. Similar observations were reported by Platt, Schulz and Kunstadter (1947).

These clinical reports would suggest that the power of the liver to inactivate oestrogen may be impaired long before the development of structural changes, such as fibrosis and necrosis.

Our experimental results lend support to this view for, on the diets used, impairment of the power of oestrogen inactivation appeared long before the occurrence of hepatic necrosis or fibrosis as evidenced by histology.

On the diet low in sulphur amino-acids and tocopherol, the inability of the liver to inactivate oestrogens occurred within 11 to 38 days, whilst in rats of the same age and the same strain, fed the same diet in this same laboratory, massive liver necrosis did not occur until 35 to 150 days (Glynn and Himsworth, 1944).

On alipotropic diets the failure to inactivate oestrogens occured as early as 7 days on the lard diet and 20 days on the starch diet. Using these same diets, the first traces of liver fibrosis were not observed until 50 days with the lard diet (Glynn, Himsworth and Lindan, 1948) and much later with starch diet (Himsworth and Lindan, unpublished data).

That oestrus could be induced in ovariectomized rats without oestrogen implants was surprising, but also has an interesting correlation with clinical findings. The feminizing changes in male patients suffering from liver disease or malnutrition have received various explanations. The presence of oestrus and of urinary output of oestrogens in ovariectomized rats fed an alipotropic diet supports the hypothesis of an extraovarian secretion of oestrogenic substances, probably by the adrenal cortex. Such oestrogens are presumably inactivated by the healthy liver.

The failure of the same diet to produce continuous oestrus in normal rats may be due to inhibiting action of other ovarian hormones. Further work will, however, be required to elucidate this point.

SUMMARY.

1. Female rats were ovariectomized and received implants of 5 mg. α-oestradiol into the spleen. Those which showed anoestrus were given deficient diets, which were known to lead either to necrosis of the liver or fatty infiltration followed by diffuse hepatic fibrosis. The effect of these diets upon the ability of liver to inactivate oestrogens was investigated.

2. Inability to inactivate oestrogens occurred long before there was evidence of hepatic necrosis or fibrosis. Supplements of the deficient factors, cystine or choline, were effective in restoring the power to inactivate oestrogens. Tocopherol supplements were ineffective within the period of this experiment.

3. Ovariectomized rats with no oestrogen implant, when given high fat diets developed permanent oestrus and oestrogenic substances were demonstrated in their urine. The existence of oestrogens other than those produced by the ovaries is indicated.

4. Clinical findings relevant to these experiments are discussed.

Grateful acknowledgment of assistance is made to Dr. H. P. Himsworth, Professor M. L. Rosenheim, Dr. O. Lindan and Dr. G. I. M. Swyer.

REFERENCES.

Biskind, G. R., and Biskind, M. S.—(1946) *Amer. J. clin. Path.*, **16**, 737.
Idem and Mark, J.—(1939) *Bull. Johns Hopk. Hosp.*, **65**, 212.
Emmens, C. W.—(1950) *J. Endocrinol.*, **6**, 302.
Glynn, L. E., and Himsworth, H. P.—(1944) *J. Path. Bact.*, **56**, 297.

Iidem AND LINDAN, O.—(1948) *Brit. J. exp. Path.*, **29**, 1.
Iidem AND NEUBERGER, A.—(1945) *Ibid.*, **26**, 326.
GYÖRGY, P.—(1945) *Proc. Soc. exp. Biol., N.Y.*, **60**, 344.
HIMSWORTH, H. P., AND GLYNN, L. E.—(1944) *Clin. Sci.*, **5**, 93.
Idem AND LINDAN, O.—(1949) *Nature*, **163**, 30.
KLATSKIN, G., SALTER, W. T., AND HUMM, F. D.—(1947) *Amer. J. med. Sci.*, **213**, 19.
LEATHES, J. B., AND RAPER, H. S.—(1925) 'The Fats.' London (Longmans, Green & Co.)
LONG, J. H., AND EVANS, H. M.—(1922) *Mem. Univ. Calif.*, **6**, 1.
PLATT, S. S., SCHULZ, R. Z., KUNSTADTER, R. H.—(1947) *Bull. U.S. Army Med. Dept.*, **7**, 403.
SEGALOFF, A.—(1945) *Proc. Soc. exp. Biol., N.Y.*, **60**, 108.
SHIPLEY, R. A., AND GYÖRGY, P.—(1944) *Ibid.*, **57**, 52.
UNNA, K., SINGHER, H. O., KENSLER, C. J., TAYLOR, H. C., AND RHOADS, C. P.—(1944) *Ibid.*, **55**, 254.

USE OF ANTIBIOTICS IN THE TREATMENT OF EXPERIMENTAL DIPHTHERIA INFECTIONS.

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ALTHOUGH effective in neutralizing the effects of the toxin when it is administered early enough, diphtheria antitoxin has no action on the bacteria which may persist in patients and in healthy carriers for a long time. Similarly, diphtheria prophylactics may protect against the development of clinical diphtheria, but do not prevent the carriage of *C. diphtheriae* in the nose, tonsils, etc. Hence it is desirable to find an agent that will clear up the infection as distinct from the toxaemia. The importance of dealing with the bacterial invasion is emphasized by the severe outbreaks experienced on the continent of Europe during the war, despite immunization campaigns, and it is again underlined in the report by Hartley, Tulloch, Anderson, Grant, Jamieson, Neubauer, Norton and Robertson (1950) on two outbreaks in this country.

Various chemotherapeutic agents are like penicillin and inhibit growth of *C. diphtheriae in vitro*, but have no effect on infections with virulent organisms in the body (Hewitt, 1948). Streptomycin was found to be an effective protection in animals against *C. diphtheriae* infections (Hewitt, 1948), but it is not an ideal agent, since it has some toxic effects and diphtheria bacilli fairly readily develop complete resistance to its effect both *in vitro* and *in vivo*. In the search for a more effective agent for the elimination of *C. diphtheriae* both in carriers and in patients, *dihydrostreptomycin*, which is less toxic, has now been investigated, and also chloromycetin and auremyocin, which are reported to be of value in infections with some organisms when administered by mouth. The potential value of some of the newer antibiotics is that they are reported also not to lead to the development of resistant strains. Apart from the practical importance of possible applications in treatment, some points of theoretical interest arise in consideration of bacterial resistance, as discussed by Selbie (1946).

EXPERIMENTAL.

Material and Methods.

The strains of *C. diphtheriae* used and the general procedures have been described in previous papers (Hewitt, 1947, 1948). The antibiotics used were dihydrostreptomycin sulphate, aureomycin and chloromycetin, together with *para*-aminosalicylic acid.

In vitro *tests.*

Serial dilutions of the antibiotics used were made in papain-digest broth and the dilutions were inoculated with 18 or 48-hour broth cultures of the organisms

investigated. Readings of the development of visible turbidity or surface pellicle were made after 48 hours' incubation.

Dihydrostreptomycin and p-*amino-salicylate.*—The minimum concentration of dihydrostreptomycin that would inhibit the growth of 9 typical *gravis*, *intermedius* and *mitis* strains of *C. diphtheriae* ranged from 0·1 to 0·4 μg. per ml. Addition of 200 μg. per ml. of *para*-aminosalicylate did not enhance the inhibitory effect.

Aureomycin and chloromycetin.—With each of the strains tested the inhibitory concentration of both aureomycin and chloromycetin was 1 or 2 μg. per ml.

Effect on animal infections.

Guinea-pigs were infected by intradermal injection of 0·2 c.c. of a broth suspension of washed virulent organisms of a *gravis* strain of *C. diphtheriae*. The antibiotic was administered either in 4 subcutaneous injections at 2-hour intervals, or by mouth in aqueous suspension in two doses with a 6-hour interval. In each case the first dose was administered immediately before infection and the last dose six hours later. Results are summarized in Table I.

TABLE I.—*Effect of Antibiotics on* C. diphtheriae *Infections in Guinea-pigs.*

Antibiotic.	Mode of administration.	Doses.	Time of death after infection (hours).
Controls	0	0	26 : 32 : 40 : 40
Chloromycetin	Subcutaneous	4 × 2 mg.	30 : 33
,,	,,	4 × 5 ,,	40 : 40
,,	Oral	2 × 100 ,,	40 : 40 : 49 : 49
Aureomycin	Subcutaneous	4 × 5 ,,	140 : 160 : 180 : 220
,,	Oral	2 × 100 ,,	77 : 110 : 160 : 160
Dihydrostreptomycin	Subcutaneous	4 × 5 ,,	S : S

S signifies alive 10 days after infection.

It will be seen from the table that chloromycetin, although inhibiting bacterial growth in culture media, has no protective effect against diphtheria infection *in vivo* either when administered subcutaneously or by mouth. Dihydrostreptomycin, like streptomycin, protects guinea-pigs against virulent *C. diphtheriae* infections when given subcutaneously in a total dosage of 20 mg.

The case of aureomycin is of some interest. The antibiotic had some irritant effect on the animals, but whilst the control animals receiving no antibiotic were all dead within 40 hours, those receiving aureomycin were all alive after 3 days, and some survived over a week. Moreover, aureomycin had a definite protective effect when given by mouth, and this raises the possibility of controlling diphtheria infections by oral dosage.

Development of bacterial resistance.

In a previous communication it was shown that serial subcultures in media containing streptomycin may diminish the sensitivity of *C. diphtheriae* to the antibiotic a thousandfold. This streptomycin-resistant strain has been sub-

cultured weekly on ordinary Loeffler slopes containing no streptomycin for six months, but its resistance to streptomycin remains unimpaired. When tested with dihydrostreptomycin it was found to be equally resistant to this antibiotic.

Aureomycin and chloromycetin.—Nine strains of *C. diphtheriae* were subcultured every 48 hours in broth containing serial dilutions of chloromycetin and aureomycin, subcultures being made from the tube showing growth which contained the highest concentration of antibiotic. In neither case, even after 40 subcultures in the presence of the antibiotic, was there any appreciable development of resistance. Growth inhibition was effected by 1 to 2 μg. per ml. in the original cultures, and in the 9 strains after 40 subcultures with the antibiotic, inhibition was still effective at a concentration of from 1 to 5 μg. per ml.

Dihydrostreptomycin.—The effect of subculturing in the presence of dihydrostreptomycin is, however, quite different. Out of the 8 strains examined, 6 developed complete resistance to the antibiotic, that is to say, growth was not inhibited by 1000 μg. per ml. of the antibiotic although the strain subcultured in broth was sensitive to 0·1 to 0·2 μg. per ml. The number of subcultures required to establish this resistance varied from 8 to 30. Parallel subcultures of each strain were made in media containing 200 μg. per ml. of *p*-aminosalicylate in addition to the graded dilutions of dihydrostreptomycin, but this had no effect on the development of resistance. Strains resistant to dihydrostreptomycin were resistant also to streptomycin.

Dependent strains.—On several occasions in the course of the subcultures it was found that organisms which had developed resistance to dihydrostreptomycin would not grow except in the presence of at least 10 μg. per ml. of the antibiotic. This dependence seemed, however, to be transitory and on subculture the organims were found to grow in the complete absence of dihydrostreptomycin. The explanation of this phenomenon is discussed later.

Toxigenicity.

After 40 subcultures in the presence of either dihydrostreptomycin alone or of dihydrostreptomycin and 200 μg. per ml. of *p*-aminosalicylate the toxigenicity of the strains in papain digest broth was determined by the method previously described (Hewitt, 1948) and the results are summarized in Table II.

TABLE II.—*Effect of Subculturing in Presence of Antibiotic on Toxigenicity.*

Strain.	Toxigenicity (units/ml.) after cultivation in: Broth.	Dihydrostreptomycin.	D.H.S. + *p*-aminosalicylate.
1 G I	0·7	1·8	1·4
34 G II	0·9	0·7	0·7
234 G II	0·7	1·5	..
265 I	2·5	3·5	2·75
13 M II	0·7	1·5	0·8
100 M VII	1·4	0·7	1·5
40 M XII	35·0	..	15
360 M XXXII	0·5	0·3	0·6

In no case had subculturing in the presence of antibiotic and the development of resistance any appreciable effect on toxigenicity.

In a further experiment strains which had developed resistance to streptomycin were grown in broth containing 100 μg. per ml. of streptomycin and the toxin contents of the filtrates were determined. The results are shown in Table III.

TABLE III.—*Effect of Presence of Streptomycin on Toxin Production by Resistant Strains.*

Strain.	Toxin production (units/ml.) in : Plain broth.	Streptomycin broth.
34 DHS	0·7	0·7
34 DHS/PAS	0·7	0·8
100 DHS	0·6	0·5
100 DHS/PAS	1·0	1·3

It will be seen that the presence of 100 μg. per ml. of streptomycin had not interfered with the toxin production by any of the strains.

Fermentation reactions.

The fermentation reactions of the strains subcultured in broth and in the presence of dihydrostreptomycin with and without *p*-aminosalicylate were identical in most cases, and the development of resistance had not affected sugar fermentation except with two strains. These two strains, which had originally belonged to the Gravis group and fermented starch, had lost this ability and were now indistinguishable from Mitis strains although, as mentioned above, their toxigenicity was not affected.

DISCUSSION.

From the point of view of immediate practical application it is of interest that dihydrostreptomycin is as effective as streptomycin (Hewitt, 1948) in protecting guinea-pigs against *C. diphtheriae* cultures. Since dihydrostreptomycin is reputed not to give the toxic reactions sometimes seen when streptomycin is used, it would seem worth while to treat both carriers of *C. diphtheriae* and infected patients with dihydrostreptomycin in order to eliminate the infecting organisms rapidly.

An interesting possibility arises that it may be possible to clear up the infection in diphtheria patients and carriers by dosage by mouth, since aureomycin had some protective effect in guinea-pigs when administered orally. Although rather less effective than injections of dihydrostreptomycin, there is a considerable advantage in being able to utilize oral dosage especially in the case of carriers who are not treated in hospital. An additional advantage in the use of aureomycin is that *C. diphtheriae* appears not to develop any appreciable resistance to the drug, so that strict supervision of initial dosage in order to avoid dissemination of resistant strains is unnecessary.

Like penicillin, chloromycetin inhibits the growth of *C. diphtheriae*, *in vitro*, but it had no effect in protecting guinea-pigs against diphtheria infection. Even after over 40 subcultures in broth containing the drugs nine strains of *C. diph-*

theriae failed to develop any appreciable resistance to either aureomycin or chloromycetin. On the other hand, resistance to dihydrostreptomycin developed after a few subcultures of six strains in media containing it, just as previously found in the case of streptomycin. Since *para*-aminosalicylate has been claimed to be of value as an adjuvant to streptomycin in the treatment of tubercular infections, it was thought possible that added to streptomycin it might delay the development of resistance to the antibiotic and enhance its action in the case of *C. diphtheriae*, but it was found to have no effect.

Some strains cultured in the presence of dihydrostreptomycin developed a dependence upon it so that they would grow only in media containing between 10 and 1000 μg. of dihydrostreptomycin per ml., but this dependence appeared to be readily lost, and dependent organisms rapidly reverted to the state of still being dihydrostreptomycin-resistant, but able to grow in its absence. The two possible explanations of this are either that adaptive enzymes are produced by the cells to effect alternative metabolic processes in the presence and absence of the antibiotic, or alternatively that the dependent cultures contain a mixed population, including mostly dependent organisms together with a few resistant cells which are capable of development in the absence of the antibiotic, and these overgrow the dependent strains under suitable conditions. It would seem that the latter explanation may be the more probable.

Strains resistant to either streptomycin or dihydrostreptomycin were found to be resistant to the other and the resistance was not lost on prolonged subculture on media not containing either antibiotic. The development of resistance following subculture in media containing the antibiotic had no deleterious effect on toxin production and the presence of streptomycin in the culture media had no effect on the production of toxin by resistant strains. As reported in a previous communication (Hewitt, 1947, 1948) some Gravis and Mitis strains of *C. diphtheriae* belong to the same serological group and are indistinguishable except in their starch-fermenting ability. In the present investigation two strains subcultured in the presence of dihydrostreptomycin and developing a high degree of resistance lost their ability to ferment starch and became Mitis strains. This lends support to the hypothesis previously advanced that the evolution of the present large number of diphtheria strains of different serological types from a common Gravis ancestor may have proceeded along the route of a Gravis strain becoming Mitis. The large variety of serological types of the Mitis group compared with Gravis strains suggests that further evolution proceeds by the development of new serological types of the Mitis strains which appear to be more variable. In this view Mitis strains may be regarded as degraded Gravis strains. No change of group occurred in the other strains investigated and Strain 360, which ferments sucrose, retained this ability after development of dihydrostreptomycin-resistance.

SUMMARY.

1. Aureomycin has some protective effect against *C. diphtheriae* infections when used orally as well as by injection. No strain was found to develop resistance to aureomycin, so that its use for treatment of diphtheria carriers by oral administration would involve the minimum of medical supervision.

2. Chloromycetin resembles penicillin in that although it is inhibitory to *C. diphtheriae in vitro* it failed to protect guinea-pigs against infection.

3. Dihydrostreptomycin protects guinea-pigs against infection with virulent *C. diphtheriae*, and it may be of value for the treatment of patients and carriers.

4. Resistance to dihydrostreptomycin develops fairly readily in the case of *C. diphtheriae* and resistant strains are resistant also to streptomycin. Such resistance is not readily lost. Resistant strains retain their toxigenicity unimpaired.

5. *Para*-aminosalicylate has no effect on the development of resistance to the antibiotic nor on its inhibitory effect.

6. Although retaining their toxigenicity unimpaired after developing dihydrostreptomycin resistance, two Gravis strains of *C. diphtheriae* lost their ability to ferment starch and became indistinguishable from Mitis strains; this lends support to the hypothesis previously advanced that Mitis strains may be degraded Gravis strains.

REFERENCES.

HARTLEY, P., TULLOCH, W. J., ANDERSON, M., DAVIDSON, W. A., GRANT, J., JAMIESON, W. M., NEUBAUER, C., NORTON, R., AND ROBERTSON, G. H.—(1950) 'A Study of Diphtheria in Two Areas in Great Britain,' M.R.C. Special Report No. 272.

HEWITT, L. F.—(1947) *Brit. J. exp. Path.*, **28**, 338.—(1948) *Ibid.*, **29**, 181, 289, 447.

SELBIE, F. R.—(1946) *Brit. med. Bull.*, **4**, 267.

STUDIES ON THE ROLE OF THE ADRENAL CORTEX IN ADENOSINE TRIPHOSPHATE SHOCK.

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SINCE Selye (1936*a*, *b*) called attention to the adrenal changes following exposure of the body to noxious agents, an ever-increasing volume of work on this subject (Selye, 1946 ; Long, 1947 ; Sayers and Sayers, 1948) has led to the development of a theory, now firmly established, that injury to the body, in any form, stimulates the hypophysis to liberate adrenocorticotrophic hormone (ACTH), which excites the secretion of adrenal cortical hormone mainly of the gluco-corticoid type. In previous work (Green, 1943 ; Bielschowsky and Green, 1943 ; Green and Stoner, 1950*a*) we have shown that adenosine triphosphate (ATP) will produce a state of shock in animals almost indistinguishable from that seen after limb ischaemia. One of the essential steps in this work was to study the behaviour of the adrenal cortex in ATP shock. The effect of ATP on the adrenal cortex has more than a passing interest, since it is not a foreign toxic agent like those usually used to induce an alarm reaction, e.g. formaldehyde, but a normal constituent of the body which may play an important part in the response to injury (Green and Stoner, 1950*a*, *b*). It has also been advocated in the treatment of rheumatoid arthritis (Lövgren, 1945), although this claim has not been confirmed (Wayne, Goodwin and Stoner, 1949).

The present study had the twofold object of determining the role of the adrenal hormones in resistance to the shock-inducing action of ATP, and the effect of ATP on the functional activity of the gland as judged by changes in its histochemistry. Whilst the precise significance of the individual histochemical tests used is not clear (Bennett, 1940 ; Weaver and Nelson, 1943 ; Popják, 1944 ; Albert and Leblond, 1946 ; Harrison and Cain, 1947 ; Yoffey and Baxter, 1947, 1949 ; Boscott and Mandl, 1949 ; Greep and Deane, 1949*a* ; Cain, 1950), by using several tests simultaneously it is possible to obtain a picture of the activity of the adrenal cortex (Dempsey and Wislocki, 1946). Furthermore, since the various zones of the adrenal cortex are separately controlled and secrete different hormones, the histochemical method possesses advantages over a purely chemical approach.

METHODS.

Three hundred and twenty albino rats, male and female, from one source were used. The sexes were used separately and not mixed in any one experiment. All operations were carried out under ether anaesthesia. Bilateral adrenalectomy was performed in one stage by the dorsal route. These animals were given 1 per cent NaCl to drink after operation, and used 3 days after removal of the

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glands. Adrenal medullectomy was done by the method of Ingle and Griffith (1942). These rats were given 1 per cent NaCl to drink for 1 week after the operation and used at the end of 3 weeks, unless stated otherwise. The completeness of the medullectomy was checked histologically at the end of the experiment. Hypophysectomy was performed by the parapharyngeal route and the rats used 3 days after the operation. The completeness of the hypophysectomy was determined at autopsy.

Ischaemic shock was produced in a few rats by applying rubber tourniquets to both hind limbs under ether anaesthesia (Rosenthal, 1943).

ATP was obtained as the Ba salt (Boots Pure Drug Co.) and converted to the Mg salt for use (Green and Stoner, 1950*a*). Paper chromatograms were made by Mr. C. J. Threlfall with this and also with muscle adenylic acid (A5MP ; Boots Pure Drug Co.), and adenosine (British Drug Houses Ltd.). These showed that the ATP contained approximately 10 per cent A5MP, whilst the other substances appeared to be pure. The control rats were given D-ribose routinely in amount and concentration equivalent to that contained in the nucleotide or nucleoside injected. A few animals were given equivalent volumes of 0·9 per cent NaCl for comparison with the ribose controls. The solutions were injected intraperitoneally at body temperature (Long, 1947), and with as little disturbance to the animal as possible. Of the cortical hormones desoxycorticosterone was used as the acetate and glucoside (Ciba Ltd.), and " Eucortone " (Allen & Hanbury, Ltd.) was used as a whole gland extract. Our thanks are due to Dr. Tindall, of Organon Ltd., for supplying us with samples of ACTH.

Toxicity tests were done on male rats fasted overnight, care being taken to ensure a constant environmental temperature during the test (Green and Stoner, 1950*a*). Rectal temperatures were recorded with a thermocouple.

Rats used for histochemical studies were killed by a blow on the head and bled from the neck. The adrenals were rapidly removed and weighed. Ascorbic acid was detected by Deane and Morse's modification (1948) of Barnett and Bourne's method (1941). Glands fixed for 48 hours in 10 per cent formol-saline and then washed in running tap water for 24 hours were used to demonstrate ketones (50μ frozen sections) by the method of Albert and Leblond (1946), and frozen sections (20μ) from the same material were examined between crossed nicol prisms or polaroid discs for the presence of birefringent material. Birefringence was also studied in frozen sections from glands fixed for 5 days at 37° C. in Bennett's (1940) formol-digitonin solution. The acetone solubility of such material was checked on several occasions. Frozen sections (20μ) from glands fixed by both methods were stained for sudanophilic lipid with Sudan III.

Plasma Mg concentrations were determined by Garner's method (1946) on samples of heart blood obtained under ether anaesthesia using heparin as an anticoagulant.

RESULTS.

Effect of adrenal hormones on the shock-inducing action of ATP.

Sensitivity to the shock-inducing action of ATP was greatly increased by adrenalectomy. The difficulties in determining the lethal dose of ATP have been discussed before (Green and Stoner, 1950*a*). In young rats of this particular strain, and under our environmental conditions (air temp. 22° C.) the LD_{100}

proved to be very high, and was between 150 to 200 mg. MgATP per 100 g. body weight (i.p.). Young animals are more resistant than older ones (Green and Stoner, 1950*a*), and this was well borne out in these experiments. After adrenalectomy the lethal dose was 10 mg. per 100 g. body weight (i.p.), and variations in toxicity due to age appeared to be eliminated. In the normal rat after a fatal dose of ATP the body temperature falls rapidly to a level close to the air temperature, where it remains for some time before death occurs (Green and Stoner, 1950*a*). Doses of ATP, without effect in the normal animal, lowered the body temperature after adrenalectomy. However, death usually occurred at a higher body temperature in the adrenalectomized rat than in the normal rat, probably because adrenalectomy decreased both the fatal dose and the survival time after such a dose. The rate of fall of the body temperature after ATP was about the same in the normal and adrenalectomized animal.

The results of toxicity tests in medullectomized rats were rather variable, but sufficient were done to show that sensitivity to the shock-inducing action of ATP was increased after this operation. Such animals often died after doses of 80 mg. MgATP per 100 g. body weight which were never fatal in the present series of normal rats, and if they survived such doses their clinical state was always worse than that of control normal rats. Hypophysectomy led to a similar, but less marked, increase in sensitivity.

Attempts to restore the resistance of the adrenalectomized rat by giving adrenal cortical hormones were not very successful. Giving 3 mg. desoxycorticosterone acetate per day (i.m.) for the 3 days after removal of the glands and 5 mg. of the glucoside (i.m.) just before injecting the ATP only raised the lethal dose to 40 mg. MgATP per 100 g. body weight. A similar increase could be obtained by giving 1·0 ml. doses of " Eucortone " in the same way, and continuing them at hourly intervals for 3 hours after the ATP injection.

Selye (1949) has described an antagonism between the mineralo-corticoids and the gluco-corticoids of the adrenal cortex, and states that the response to various stress-inducing agents is increased by the former and reduced by the latter. Whilst we found a slightly increased response to limb ischaemia after pre-treatment with large doses of desoxycorticosterone, this substance appeared to have little effect on the response to ATP. It was given either in daily doses of 3 mg. of the acetate (i.m.) for 4 days followed by a single dose of 5 mg. of the glucoside just before the experiment, or as 3-mg. doses of the acetate (i.m.) just before and 4 hours after the injection of the ATP, and in neither case was the response significantly altered. " Eucortone " had a slight therapeutic action. When given in 1·0 ml. per 100 g. body weight doses (i.m.) just before and again at 2 and 4 hours after the injection of ATP it improved the clinical state of the animal whilst not affecting the mortality rate. This effect has also been seen in mice.

Effect of ATP on the histochemistry of the adrenal cortex.

In most experiments a dose of ATP (75 to 100 mg. MgATP per 100 g. body weight) was chosen which would give a moderately severe, but non-fatal, state of shock as judged by the body temperature (Fig. 1). The combined adrenal weight was determined in 66 male rats killed at varying intervals up to 24 hours after this dose, and compared with that in 52 male rats killed at similar intervals

after the injection of an equivalent amount of D-ribose. D-ribose caused no apparent disturbance in the animals, and when their combined adrenal weights, expressed as mg. per 100 g. body weight, were plotted against the body weight, the points fell on a curve similar to that shown by Andersen and Kennedy (1932), illustrating the reciprocal relationship between adrenal weight and body weight. The combined adrenal weights of the ATP-treated rats, expressed in the same way, fell about the same curve.

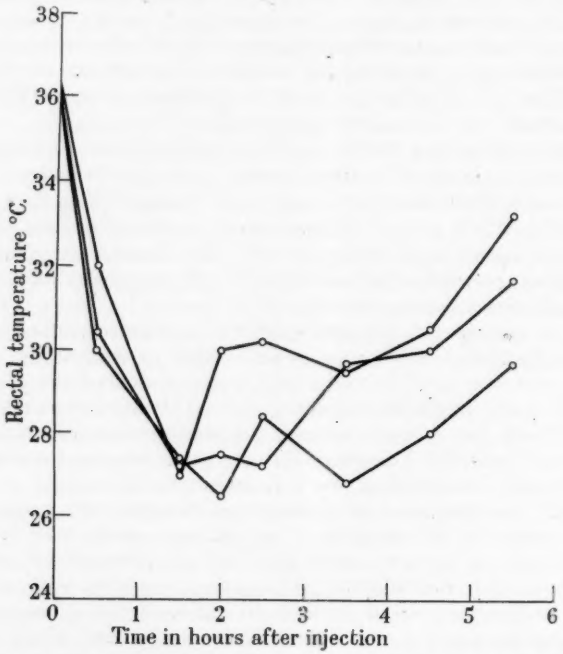


FIG. 1.—The effect of non-lethal doses of ATP on the rectal temperature. Each curve shows the changes in the rectal temperature of a fasting rat given 75 mg. MgATP per 100 g. body weight (i.p.) at zero time.

In the haematoxylin and eosin counterstained paraffin sections from the glands stained for ascorbic acid the adrenal cortex appeared hyperaemic after ATP. There was also an increase in the size of the cells of the zona fasciculata, which was maximal 7 hours after the injection (Fig. 3 and 4). This increase in cell size is of interest in view of the absence of any overall change in weight and the loss of much of the contents of these cells (v.i.). The change is probably due to an increase in their water content (Popják, 1944). The average water content of the whole adrenal gland 7 hours after this dose of ATP was 74·3 per cent, which was slightly higher than in the controls (73·6 per cent), but the difference was not statistically significant. Estimations of the fat free dry weight (Hastings and Eichelberger, 1937) showed that there was a significant decrease in the fat content of the adrenal at this time after the ATP injection.

The number and distribution of the rats receiving a non-fatal dose of ATP whose adrenals were examined histochemically is given in Table I.

TABLE I.—*Shows the Number of Rat Adrenals Examined Histochemically at Different Intervals after the Injection of a Non-fatal Dose of ATP.*

Time (hr.) after i.p. non-fatal dose of ATP (exp.) or an equivalent amount of d-ribose (control).	Histochemical test. Ascorbic acid. Exp. ♂	Ascorbic acid. Exp. ♀	Ascorbic acid. Control ♂	Ascorbic acid. Control ♀	Sudanophilia. Exp. ♂	Sudanophilia. Exp. ♀	Sudanophilia. Control. ♂	Sudanophilia. Control. ♀	Birefringence. With digitonin. Exp. ♂	Birefringence. With digitonin. Exp. ♀	Birefringence. With digitonin. Contr. ♂	Birefringence. With digitonin. Contr. ♀	Birefringence. Without digitonin. Exp. ♂	Birefringence. Without digitonin. Exp. ♀	Ketones. Exp. ♂	Ketones. Con. ♀
1	5	4	3	4	6	1	5	1	6	1	4	1	3	2	4	2
4	5	2	3	2	18	2	6	2	9	2	3	2	8	5	6	4
7	3	–	3	–	6	–	4	–	5	–	4	–	3	2	2	2
24	3	3	3	3	5	3	3	3	5	3	3	3	–	–	1	1

After a non-fatal dose of ATP the first histochemical change in the adrenal cortex was a fall in its ascorbic acid content. Control rats, given D-ribose, showed numerous argentophil granules spread uniformly in the cytoplasm, being densest in the cells of the zona fasciculata (Fig. 2). There were few granules in the cells of the zona glomerulosa and in the zona reticularis, their number diminished as the medulla was approached. The picture in these rats remained the same throughout the 24-hour period studied, and closely resembled that given for the normal rat by Deane and Morse (1948). After ATP the number and size of the granules in the cells of the zona fasciculata decreased progressively, reaching a minimum 7 hours after the injection (Fig. 3 and 4). Argentophil granules are normally present between the cells in the sinusoids, indicating an early stage in secretion (Deane and Morse, 1948). This was very prominent in glands examined 1 and 4 hours after the injection of ATP (Fig. 5). The ascorbic acid content of the zona reticularis fell, but the granules in the zona glomerulosa were unaffected by ATP. By 24 hours normal conditions had been restored, and there was now no difference between the treated and control animals.

Lipid distribution in the normal rat adrenal cortex is rather variable (Harrison and Cain, 1947 ; Cain and Harrison, 1950). In our control material one usually saw a densely packed zona glomerulosa, separated by a narrow, relatively sudanophobic zone from the fairly uniformly dense zonae fasciculata and reticularis where the density of lipid decreased near the medulla (Fig. 6). After ATP definite changes in the amount of sudanophilic material were not seen till 4 hours after the injection. In glands taken at 4 hours (Fig. 7 and 8) and 7 hours there was always considerably less than in the control glands. The loss of lipid was confined to zonae fasciculata and reticularis, where the change was comparable to that produced by the injection of between 1 and 3 mg. ACTH (Fig. 9). Complete recovery occurred in 24 hours.

The phenylhydrazone-forming material of the normal adrenal cortex is concentrated chiefly in the zona glomerulosa and outer part of the zona fasciculata. After ATP the ketone material in the zona fasciculata was reduced, whilst that in the zona glomerulosa remained unchanged. The time relations of these changes were the same as for the sudanophilic material.

EXPLANATION OF PLATES.

Plate I (Fig. 2–5).

High power (× 700) photomicrographs of the zona fasciculata of the rat adrenal cortex after treatment with $AgNO_3$ to demonstrate ascorbic acid.

Fig. 2.—Rat (142 g. body weight, ♂) killed 1 hour after the injection of a dose of D-ribose equivalent to 75 mg. MgATP per 100 g. body weight. The section shows the normal distribution of the argentophil granules in the cytoplasm.

Fig. 3.—Rat (124 g. body weight, ♂) killed 1 hour after the injection (i.p.) of 90 mg. MgATP per 100 g. body weight.

Fig. 4.—Rat (134 g. body weight, ♂) killed 7 hours after the injection (i.p.) of 75 mg. MgATP per 100 g. body weight.

Fig. 3 and 4 show the reduction in the number and size of the granules and the increase in the size of the cells after ATP.

Fig. 5.—Rat (178 g. body weight, ♀) killed 1 hour after the injection (i.p.) of 75 mg. MgATP per 100 g. body weight. The granules are seen to be lying in the sinusoids between the cells showing an early stage in secretion.

Plate II (Fig. 6–9).

Low-power (× 75) photomicrographs of the male rat adrenal cortex treated with Sudan III.

Fig. 6.—Rat (116 g. body weight) killed 4 hours after the injection (i.p.) of a dose of D-ribose equivalent to 95 mg. MgATP per 100 g. body weight. The section shows the normal distribution of sudanophilic lipid in the zones of the cortex.

Fig. 7.—Rat (139 g. body weight) killed 4 hours after the injection (i.p.) of 75 mg. MgATP per 100 g. body weight.

Fig. 8.—Rat (154 g. body weight) killed 4 hours after the injection (i.p.) of 75 mg. MgATP per 100 g. body weight.

Figs. 7 and 8 show the decrease in the sudanophilia of the zonae fasciculata and reticularis after ATP, with the retention of lipid in the zona glomerulosa.

Fig. 9.—(a) Rat (95 g. body weight) killed 4 hours after the injection (i.p.) of 3 mg. ACTH. (b) Rat (110 g. body weight) killed 7 hours after the injection (s.c.) of 3 mg. ACTH.

The sections show the discharge of sudanophilic lipid from the zonae fasciculata and reticularis.

Plate III (Fig. 10).

Low-power (× 70) photomicrographs of the male rat adrenal cortex fixed in formol-digitonin and examined between crossed polaroid discs. The white line indicates the position of the capsule.

The lower series is from rats given 75 mg. MgATP per 100 g. body weight (i.p.) and the upper from rats given an equivalent amount of D-ribose. The numbers on the photographs show the interval (hours) between the injection and the killing of the animal.

The sections show the great decrease in the birefringence of the zona fasciculata after ATP administration. The apparent changes in the zona glomerulosa are artefacts (see text).

Plate IV (Fig. 11 and 12).

Fig. 11.—Low power (× 70) photomicrograph of the male rat adrenal cortex fixed in formol-digitonin. Rat (110 mg. body weight) killed 7 hours after the injection (s.c.) of 3 mg. ACTH. The white line indicates the position of the capsule. This section should be compared with those in Fig. 10 taken from ATP-treated rats.

Fig. 12.—Low-power (× 75) photomicrographs of the male rat adrenal cortex treated with Sudan III. The sections show the increase in the sudanophilic lipid of the cortex after repeated treatment with ATP (b) as compared with the normal density of the control (a). See text for details.

Plate V (Fig. 13).

Photomicrographs of the adrenal cortex from a female hypophysectomized rat which died 4¼ hours after the injection (i.p.) of 110 mg. MgATP per 100 g. body weight.

Fig. 13a.—Fixed in 10 per cent formol-saline and treated with Sudan III. (× 75.)

Fig. 13b.—Fixed in formol-digitonin and examined, unstained, between crossed polaroid discs (× 70). The white line indicates the position of the capsule.

The sections show the retention of sudanophilic lipid and birefringent material after the injection of ATP in the hypophysectomized animal.

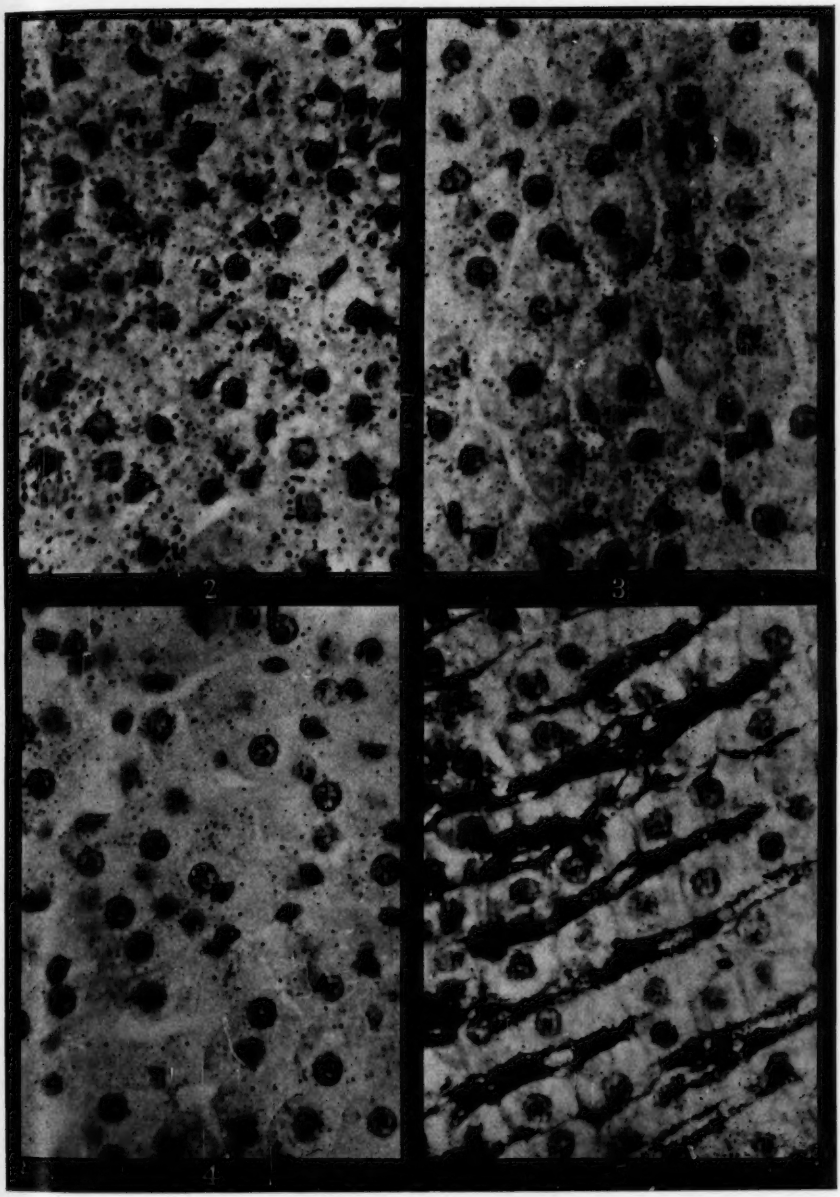
Plate VI (Fig. 14).

Photomicrographs of the male rat adrenal 3 weeks after medullectomy, and fixed in 10 per cent formol-saline.

Fig. 14a.—Rat (148 g. body weight) which died 3¼ hours after the injection (i.p.) of 150 mg. MgATP per 100 g. body weight. Treated with Sudan III. (× 75.)

Fig. 14b.—Rat (152 g. body weight) which died 6 hours after the injection (i.p.) of 90 mg. MgATP per 100 g. body weight. Examined unstained between crossed polaroid discs (× 70). The white line indicates the position of the capsule.

The sections show the persistence of the sudanophilic lipid and birefringent material after ATP in the adrenal medullectomized rat.



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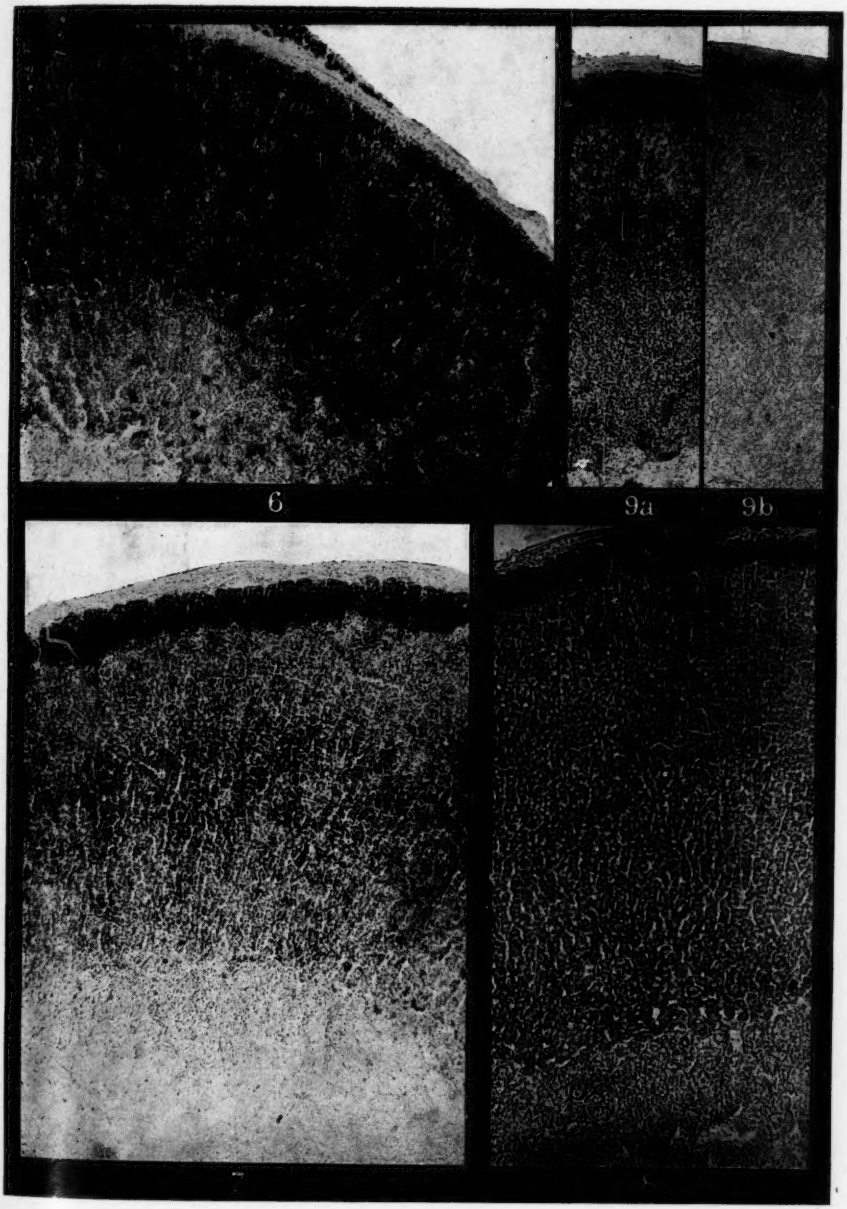

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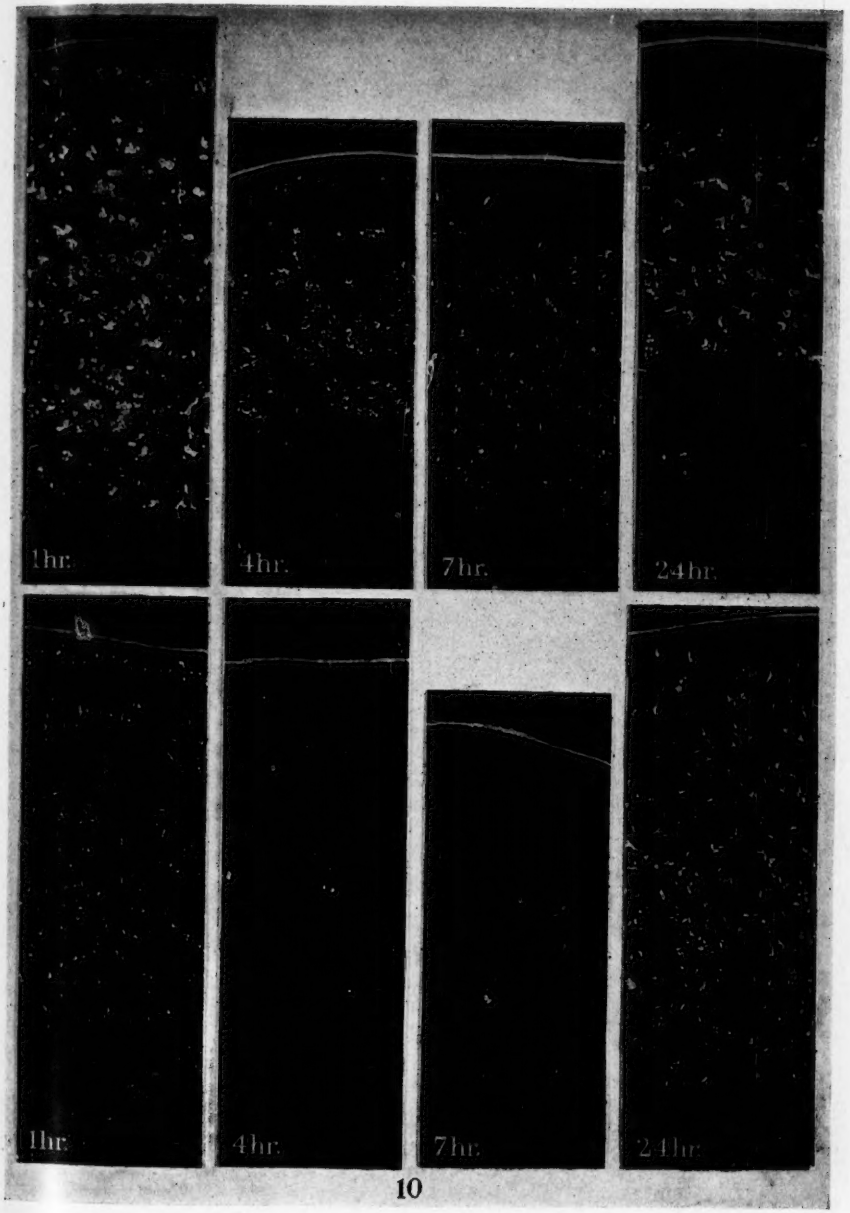

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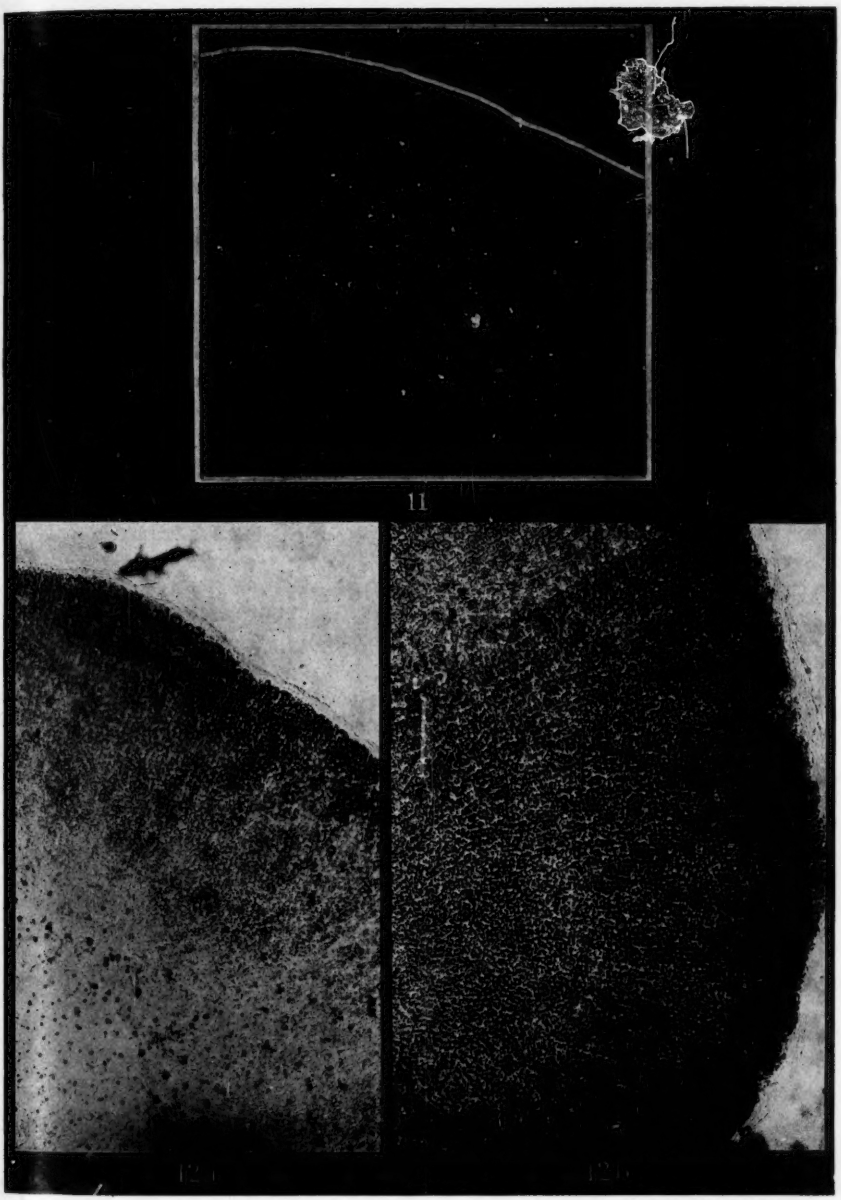

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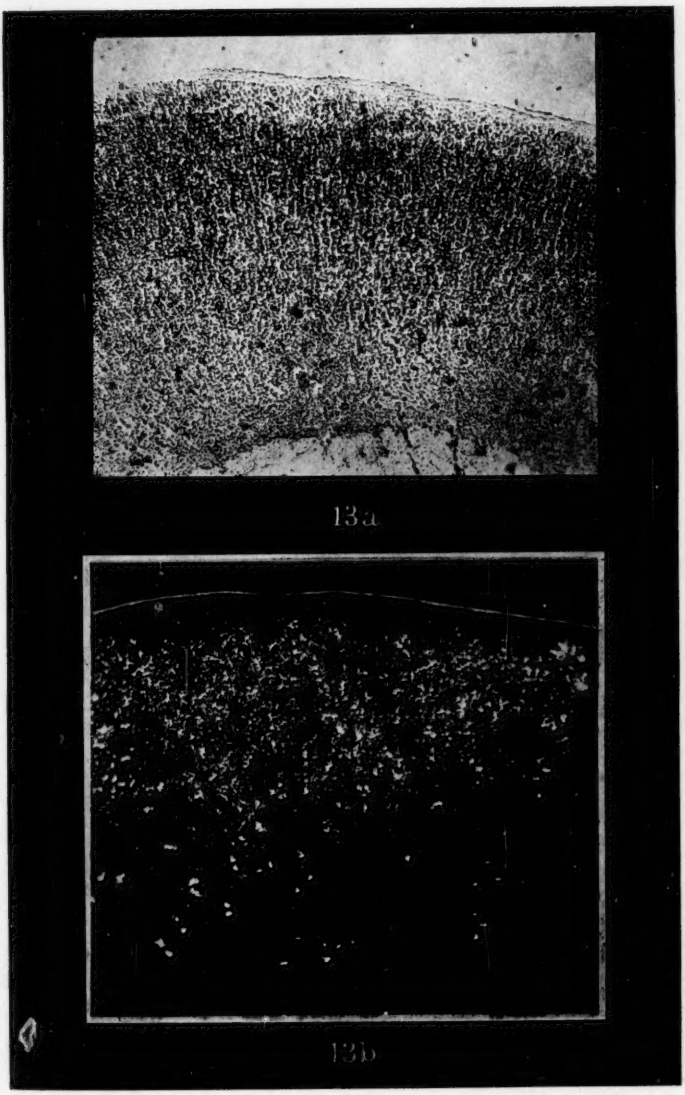

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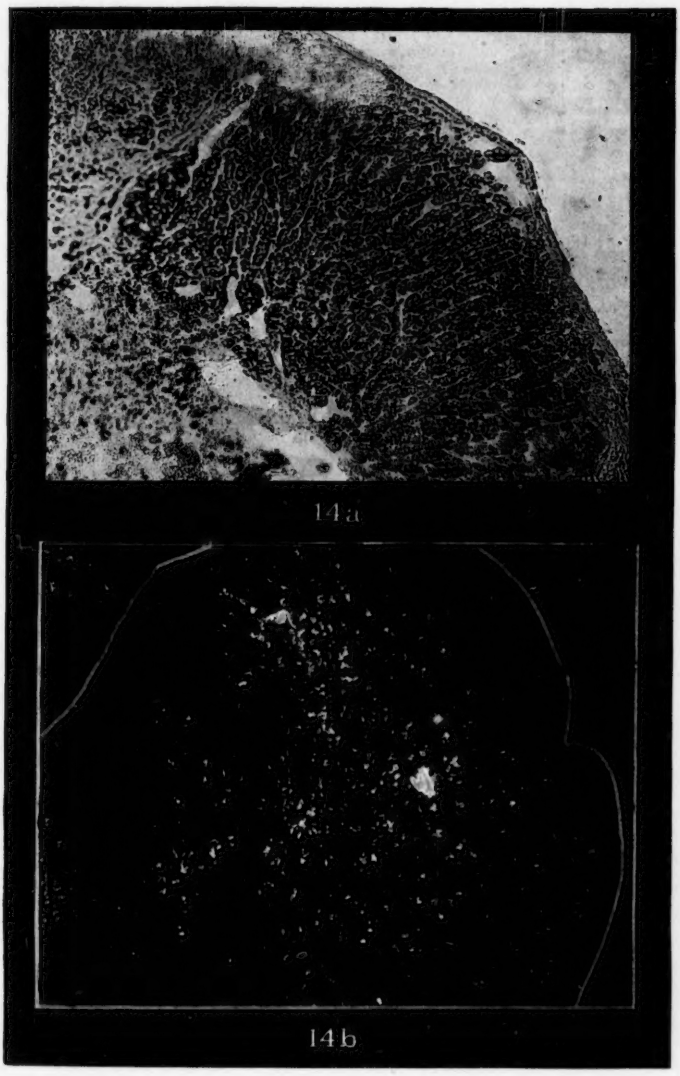

14a

14b

When the adrenal cortex is examined with polarized light droplets containing ketosteroid exhibit birefringence, which may be increased by treating the glands with digitonin to form insoluble anisotropic esters with cholesterol and other β-steroids. Large birefringent particles are associated with storage, whilst small ones are thought to indicate active secretion (Greep and Deane, 1949*a*). In ATP-treated rats both the number and size of the particles was greatly reduced, reaching a minimum 7 hours after the injection (Fig. 10). The decrease was comparable to that seen after the subcutaneous injection of 3 mg. ACTH (Fig. 11). Both with ACTH and ATP the loss of birefringent material was from the cells of the zona fasciculata. The apparent changes in the zona glomerulosa seen in the photomicrographs are artefacts due to the difficulties of focusing, etc. ; the birefringence of that zone was, in fact, unaltered. Recovery of the birefringent material occurred within 24 hours when, in fact, the zona fasciculata of the ATP-treated rat usually showed more birefringence than the control (Fig. 10).

These effects of ATP on the histochemistry of the adrenal cortex were seen in both male and female rats, and both in fed animals and those which had been fasted overnight.

Repeated exposure to stress usually leads to adrenal hypertrophy in the first instance. Three rats given 95 mg. MgATP per 100 g. body weight on alternate days for 3 doses were killed 36 hours after the last injection, and their adrenals compared with those from 3 rats similarly treated with equivalent amounts of D-ribose. Although the adrenals from the ATP-treated rats were not significantly heavier than normal they were yellower in colour. The zonae fasciculata and reticularis contained much more sudanophilic lipid than the controls (Fig. 12), and in the zona fasciculata there was more ketone and more birefringent material, chiefly in the form of large masses.

Effect of fatal doses of ATP.

When fatal doses of ATP were given intraperitoneally and the adrenals examined at death, or when the animal was moribund, the histochemical changes described above were exaggerated. Under these conditions the zona fasciculata was practically denuded of sudanophilic, ketone and birefringent material, whilst the zona glomerulosa appeared normal.

Effect of fatal limb ischaemia.

Fatal ischaemic shock was produced by occluding the circulation to both hind limbs for 5 hours. There was little change in the histochemistry of the adrenal cortex during the period of ischaemia, but 7 hours after release of the occlusion the gland presented a similar picture to that seen at the same time after a non-fatal dose of ATP. As before, the changes were confined to the zonae fasciculata and reticularis, but they did not occur as rapidly as after a fatal dose of ATP, nor was the reduction in ascorbic acid so great.

Effect of ATP after hypophysectomy.

To determine the role of the hypophysis in the adrenal response to ATP it was given, in doses within the non-fatal range for normal animals, to 6 rats 3 days after complete hypophysectomy, i.e. before adrenal atrophy had occurred and

whilst the gland appeared superficially normal. In these animals the zona fasciculata retained its histochemical properties after ATP administration, and the marked discharge of ketosteroid material was not observed (Fig. 13). Rats in which the skull was opened but the hypophysis not removed behaved normally after the injection of ATP.

Effect of ATP after adrenal medullectomy.

The effect on the adrenal cortex of normally non-fatal doses of ATP was studied in 15 rats 21 days after removal of the adrenal medullae and in 5 rats 47 days after this operation. ATP appeared to have little effect on the adrenal cortex of these animals (Fig. 14). Even in rats dying after such a dose of ATP the ascorbic acid content, sudanophilia and birefringence of the zona fasciculata appeared normal. This difference between the normal and medullectomized animal is interesting, since Greep and Deane (1949*b*) have shown that the regenerated gland after enucleation has an apparently normal secretory function, and we have found that it responds in the normal way to the subcutaneous injection of ACTH.

Effect of nucleotide derivatives.

To study the mechanism of the ATP effect some of its derivatives were also tested. Compounds were compared with a non-lethal dose of ATP by injecting them intraperitoneally in equimolecular amounts. Given in this way A5MP caused some discharge of sudanophilic and birefringent material from the zona fasciculata, but was much less active then ATP. Adenosine did not alter the appearance of the glands. As already described, D-ribose was used for control purposes, and had no effect on the glands when compared with equivalent volumes of 0·9 per cent NaCl.

Effect of sodium pyrophosphate.

The effect of $Na_4P_2O_7$ (0·1M) in amounts equivalent to non-fatal doses of ATP, on the basis of pyrophosphate content, was observed in 10 rats. Unlike previous series (Bielschowsky and Green, 1944; Green and Stoner, 1950*a*) the strain of rats used in these experiments was more susceptible to $Na_4P_2O_7$ than ATP, so that this dose was either fatal or produced a more severe state of shock than equivalent amounts of ATP. Despite this $Na_4P_2O_7$ did not cause as much histochemical change in the adrenal cortex as ATP. Four hours after the injection the sudanophilia and birefringence of the zona fasciculata appeared normal in 3 of 4 rats. In rats dying after $Na_4P_2O_7$ the ascorbic acid, sudanophilia, birefringence and ketones of this zone were reduced, but less strikingly than in rats examined 7 hours after a non-lethal dose of ATP.

DISCUSSION.

If we accept the various histochemical tests used as giving a fair picture of the functional activity of the adrenal cortex, our results show that ATP shock is accompanied by secretion from the zona fasciculata, and that repeated administration of ATP leads to increased ketosteroid storage in that zone. The effect of ATP on the adrenal cortex, therefore, closely resembles that of other stress-inducing agents and of ACTH. Of the adenyl compounds tested ATP was the

most active, activity being directly related to the shock-inducing potency of the compound. In this connection the results obtained with $Na_4P_2O_7$ are interesting, for whilst the degree of shock was greater than that produced by equivalent amounts of ATP, the adrenal cortical changes were very much less. In earlier work (Bielschowsky and Green, 1944 ; Green and Stoner, 1950*a*) we have tended to consider the shock-inducing actions of ATP and $Na_4P_2O_7$ as being closely related, and to draw the conclusion that this action of ATP is related to its phosphate groups. Whilst other evidence supports this conclusion (Green and Stoner, 1950*a*), the results described above and others obtained recently (Stoner, Green and Threlfall, 1950) show that this similarity is not as great as originally thought. Possible explanations for the smaller effect of $Na_4P_2O_7$ on the cortex such as the inhibition of local enzymic mechanisms associated with secretion have not yet been explored.

The action of ATP on the adrenal cortex of the intact rat is not a direct one, but depends upon the secretions of two other endocrine glands. Although in experiments on the perfused gland Vogt (1949) found that the addition of ATP to the perfusion fluid increased the output of cortical hormone, she considered that this was more probably due to the restoration of normal functioning conditions than to active stimulation. Whilst our methods cannot completely exclude any direct action on the gland, the results provide no evidence for it, and that is certainly not the main pathway of the effect in the intact animal. The experiments on the hypophysectomized and medullectomized rats suggest the following series of events. The injection of ATP first causes the secretion of adrenaline, which in turn leads to the liberation of ACTH from the anterior lobe of the hypophysis, and hence to the changes in the adrenal cortex. Our studies on carbohydrate metabolism in ATP shock (Stoner *et al.*, 1950) provide additional evidence that adrenaline is released in this condition and in amounts sufficient to cause a striking hyperglycaemia, i.e. in amounts of the order required for stimulation of the hypophysis (Ronzoni and Reichlin, 1950). The activity of the other adenyl compounds is in the same order as their hyperglycaemic action. Although it is agreed that both adrenaline and nor-adrenaline will discharge hormones from the adrenal cortex (Vogt, 1944 ; Long and Fry, 1945 ; Long, 1947 ; Naysmyth, 1950), the mechanism of the effect is disputed. Long and his co-workers tend to the view that adrenaline acts through the hypophysis (Long, 1949 ; McDermott, Fry, Brobeck and Long, 1950), whilst Vogt considers that adrenaline can act directly on the adrenal cortex, although she admits that adrenal hypertrophy following repeated doses of adrenaline is dependent upon the hypophysis (Vogt, 1945). Again, it is questionable if the methods we have used are sufficiently sensitive to determine whether or not the adrenaline liberated under these conditions can act directly on the adrenal cortex. Our results show, however, that, as envisaged by Long, the main pathway lies through the hypophysis.

These results may throw some light on the controversy which has arisen over the mechanism of ACTH liberation under conditions of stress. According to Long (1947, 1949) adrenaline forms the link between the stress-inducing agent and the hypophyseal-adrenal cortical response. Sayers and Sayers (1947, 1948), on the other hand, claim that a state of stress in the body leads to greater utilization of cortical hormone, and that the increased demand for hormone leads to the liberation of ACTH and stimulation of the adrenal cortex. Whilst some

mechanism of this sort probably exists (Greep and Deane, 1949*b*), it does not seem to account for the response in stress. Since the increased demand for cortical hormone in ATP shock is presumably as great in the medullectomized rat as in the normal rat, the lack of histochemical response and the greater sensitivity of the former is strong evidence in favour of Long's theory that adrenaline is an essential link in the chain.

Whilst the relationships between the endocrine glands involved in the response to stress become clearer, the gap between the stress-inducing agent and the commencement of that response remains unbridged. Since evidence exists that the adenine nucleotides are concerned in the general bodily reaction to injury (Green and Stoner, 1950*a*, *b*) our present results suggest possible bridges for that gap, but until the extent of their involvement is accurately defined it would be unwise to proceed further with such an hypothesis.

Leaving these considerations aside there is no doubt that, as in other forms of shock (Swingle and Parkins, 1935), the functional state of the adrenal gland is an important factor in determining the outcome of ATP shock. Anything which interferes with the normal discharge of cortical hormones, such as removal of the glands or their inactivation by medullectomy or hypophysectomy, greatly increases the animal's sensitivity to ATP. The relatively greater size of the adrenal in the young animal (Korenchevsky, 1942 and *v.s.*) may partly account for their greater resistance to the shock-inducing action of ATP, especially since the effect of age on sensitivity was not seen after adrenalectomy.

The limitation of the histochemical response to the zona fasciculata suggests that in ATP shock, as in shock from tissue injury, the protective action of the adrenal cortex is exerted through its gluco-corticoid hormones, and that it is their removal which sensitizes the adrenalectomized animal to this stress. A further possibility must, however, be considered, especially in view of the poor response to replacement therapy with either type of hormone. Conway and Hingerty (1946) have shown that in adrenalectomized rats, not maintained on NaCl, and in fairly advanced stages of deficiency, the Mg content of the plasma and muscle is raised. Since Mg^{++} administration greatly increases the toxic effects of ATP and its derivatives (Green and Stoner, 1944, 1950*a*), we determined the plasma Mg content in adrenalectomized rats under our conditions i.e., maintained on NaCl, deprived of food overnight, and apparently in good health, and found it to be within the normal range. Consequently this mechanism would not seem to be concerned in our experiments. The explanation of the poor response to replacement therapy probably lies in the differing potencies of the hormone preparations used. Desoxycorticosterone acetate is a pure substance with marked physiological activity so that its slight effect when given in large doses is hardly striking evidence in favour of the involvement of the mineralo-corticoids in the response, especially in view of its possible conversion to glycogenic material in the body (Hayano, Dorfman and Prins, 1949). In comparison the gluco-corticoid potency of a whole gland extract is low, which increases the significance of its similar effect to desoxycorticosterone acetate in the adrenalectomized animal and its therapeutic effect in the normal one. A valid comparison would be between desoxycorticosterone and cortisone, but supplies of the latter were not available to us.

The mechanism of the protective action of the gluco-corticoids in ATP shock is not clear, nor indeed has a satisfactory explanation been advanced in any form

of shock. Work on this problem is proceeding, and the results will be embodied in a further paper.

SUMMARY.

The role of the adrenal cortex in nucleotide shock has been studied in the rat. It was found that ATP acts like other shock-inducing agents in causing the discharge of hormones from the zona fasciculata of the adrenal cortex as judged by histochemical tests. Derivatives of ATP have a similar effect in proportion to their shock-inducing potency, but $Na_4P_2O_7$ is not nearly as effective as ATP. The effect is not a direct one on the adrenal cortex, but is mediated through the adrenal medulla and hypophysis. The prevention of the response by adrenalectomy, adrenal medullectomy or hypophysectomy increases the animal's sensitivity to ATP. The protective action of the adrenal cortex is thought to be due to its gluco-corticoid hormones. The results are discussed in relation to current theories on the mechanism of the adrenal response to body injury.

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REFERENCES.

ALBERT, S., AND LEBLOND, C. P.—(1946) *Endocrinology*, **39**, 386.
ANDERSEN, J. H., AND KENNEDY, H. S.—(1932) *J. Physiol.*, **76**, 247.
BARNETT, S. A., AND BOURNE, G.—(1941) *J. Anat.*, **75**, 251.
BENNETT, H. S.—(1940) *Amer. J. Anat.*, **67**, 151.
BIELSCHOWSKY, M., AND GREEN, H. N.—(1943) *Lancet*, ii. 153. —(1944) *Nature*, **153**, 524.
BOSCOTT, R. J., AND MANDL, A. M.—(1949) *J. Endocrinol.*, **6**, 132.
CAIN, A. J.—(1950 *Biol. Rev.*, **25**, 73.
Idem AND HARRISON, R. G.—(1950) *J. Anat.*, **84**, 196.
CONWAY, E. J., AND HINGERTY, D.—(1946) *Biochem. J.*, **40**, 561.
DEANE, H. W., AND MORSE, A.—(1948) *Anat. Rec.*, **100**, 127.
DEMPSEY, E. W., AND WISLOCKI, G. B.—(1946) *Physiol. Rev.*, **26**, 1.
GARNER, R. J.—(1946) *Biochem. J.*, **40**, 828.
GREEN, H. N.—(1943) *Lancet*, ii, 147.
Idem AND STONER, H. B.—(1944) *Brit. J. exp. Path.*, **25**, 150.—(1950*a*) 'Biological Actions of the Adenine Nucleotides.' London (Lewis).—(1950*b*) *Brit. med. J.*, i, 805.
GREEP, R. O., AND DEANE, H. W.—(1949*a*) *Ann. N.Y. Acad. Sci.*, **50**, 596.—(1949*b*) *Endocrinol.*, **45**, 42.
HARRISON, R. G., AND CAIN, A. J.—(1947) *J. Anat.*, **81**, 286.
HASTINGS, A. B., AND EICHELBERGER, L.—(1937) *J. biol. Chem.*, **117**, 73.
HAYANO, M., DORFMAN, R. I., AND PRINS, D. A.—(1949) *Proc. soc. exp. Biol. N.Y.*, **72**, 700.
INGLE, D. J., AND GRIFFITH, J. Q.—(1942) 'The Rat in Laboratory Investigation (Griffith, J. Q., and Farris, E. J.). Philadelphia (J. B. Lippincott Co.)
KORENCHEVSKY, V.—(1942) *J. Path. Bact.*, **54**, 13.
LONG, C. N. H.—(1947) *Recent Progress in Hormone Research*, **1**, 99.—(1949) 'The Chemistry and Physiology of Growth.' (Princeton University Press), p. 266.
Idem AND FRY, E. G.—(1945) *Proc. soc. exp. Biol. N.Y.*, **59**, 67.
LÖVGREN, O.—(1945) *Acta med. Scand. Suppl.*, **163**.
MCDERMOTT, W. V., FRY, E. G., BROBECK, J. R., AND LONG, C. N. H.—(1950) *Proc. soc. exp. Biol. N.Y.*, **73**, 609.

NAYSMYTH, P. A.—(1950) *J. Physiol.*, **110**, 294.
POPJÁK, G.—(1944) *J. Path. Bact.*, **56**, 485.
RONZONI, E., AND REICHLIN, S.—(1950) *Amer. J. Physiol.*, **160**, 490.
ROSENTHAL, S. M.—(1943) *Publ. Hlth. Rep., Wash.*, **58**, 1429.
SAYERS, G., AND SAYERS, M. A.—(1947) *Endocrinology*, **40**, 265.—(1948) *Recent Progress in Hormone Research*, **2**, 81.
SELYE, H.—(1936*a*) *Nature*, **138**, 32.—(1936*b*) *Brit. J. exp. Path.*, **17**, 234.—(1946) *J. Allergy*, **17**, 231, 289, 358.—(1949) *Brit. med. J.*, ii, 1129.
STONER, H. B., GREEN, H. N., AND THRELFALL, C. J.—(1950) 'Proc. XVIII International Physiological Congress,' p. 473.
SWINGLE, W. W., AND PARKINS, W. M.—(1935) *Amer. J. Physiol.*, **111**, 426.
VOGT, M.—(1944) *J. Physiol.*, **103**, 317.—(1945) *Ibid.*, **104**, 60.—(1949) *Fed. Proc.*, **8**, 341.
WAYNE, E. J., GOODWIN, J. F., AND STONER, H. B.—(1949) *Brit. Heart J.*, **11**, 55.
WEAVER, H. M., AND NELSON, W. O.—(1943) *Anat. Rec.*, **85**, 51.
YOFFEY, J. M., AND BAXTER, J. S.—(1947) *J. Anat.*, **81**, 335.—(1949) *Ibid.*, **83**, 89.

THE EFFECT OF INJECTION OF DIPHTHERIA PROPHYLACTIC INTO APPARENTLY NORMAL HORSES.

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FOR many years there has been a gradual decline in the number of normal horses possessing diphtheria antitoxin acquired as a result of natural stimulation from infection, and during the recent war years this situation led to difficulty in obtaining enough suitable horses for the production of diphtheria antitoxin for therapeutic use. It therefore became necessary to consider methods by which basal immunity could best be established artificially in incoming horses. The presence of normal antitoxin is of great importance in serum production, because the long delays necessary for the artificial establishment of basal immunity in non-immune animals are avoided and hyperimmunization can be started at once. The significance of the diphtheria antitoxic content of normal horses and its relation to successful hyperimmunization has been discussed by Barr and Glenny (1945), who showed that 28 per cent of horses with a normal value below 0·01 unit and 80 per cent of those with a normal value of 0·2 unit per ml. yielded antitoxic titres of 800 units or more in a preliminary course of hyperimmunization with toxoid. This conclusion was reached from the analysis of the results of immunization of 450 horses, and the value 800 units per ml. was taken as the lower limit of successful immunization in the first course of injections. In the production of tetanus antitoxic serum, and indeed all antitoxins which are not developed in the horse from chance infections, a course of preliminary injections is necessary, followed by a long rest lasting several months, if high titre antitoxin of good avidity is to be produced by hyperimmunization.

Table I shows the percentage number of incoming horses bought by these Laboratories, that contained detectable circulating diphtheria antitoxin (over

TABLE I.—*Showing the Numbers of Normal Horses with Detectable Circulating Diphtheria Antitoxin (over 0·0005 unit/ml.) at Intervals during the last 26 years.*

Period reviewed.	Number of horses tested.	Number with detectable antitoxin.	Percentage with detectable antitoxin.
1924–27	772	551	71·4
1932–35	1939	1024	52·8
1940–43	3439	1574	45·8
1949, 1950	400	67	16·7

0·0005 unit per ml.) over 4-year periods at intervals over the last 26 years. These figures do not include any horses known to have been purchased from the Army, or to have come from any country other than Great Britain, because it was found

(Barr and Glenny, 1945) that Army and Polish horses had a very high normal immunity rate to diphtheria ; it was also found (unpublished) that few horses from Ireland had detectable naturally-acquired antitoxin. Table I shows that there were proportionally more than four times as many horses with antitoxin arriving at these Laboratories in the period 1924–27 as there were among those entering in 1949, 1950. There has been a steady decline in the incidence of detectable antitoxin over the period reviewed, but the greatest falls appear to have occurred between the first two and the last two periods in the table. The reason for the earlier fall is not apparent, but the subsequent fall since 1943 may be due to the absence of serious epidemics and the reduction in the carrier rate of the population during the life of the horses arriving at these Laboratories in recent years.

Establishment of basal immunity in normal horses.

All incoming horses to the Laboratories were injected with a dose of 5 ml. of diphtheria A.P.T. in order to determine by tests on blood samples taken 10 days later which animals had some degree of potential immunity. Some of these would be expected to respond better to hyperimmunization than those with no potential immunity, although, as suggested by Barr and Glenny (1945), it is probable that few horses with no circulating antitoxin possess very good basal immunity. In order to increase the degree of the latter in the potentially immune horses and to establish it in those previously non-immune, a second injection of A.P.T. was given 28 days after the first. Blood samples were taken 10 days after the first injection, at the time of the second injection and 14 days later. These were tested for antitoxic content by the guinea-pig intracutaneous method, tests being made at approximately 100 per cent differences, because the very great scatter of values rendered closer testing unnecessary for the purpose of determining the presence or absence of potential immunity before injection.

Variables in the immunological state of normal horses.

It is clear on theoretical grounds that so-called normal horses (i.e. horses which have received no injections of specific antigen) can be classified in a number of groupings according to their natural immunological history, and that this earlier history would influence their subsequent behaviour after artificial stimulation.

Horses that had had considerable contact with infection would have developed circulating antitoxin, the amount of this depending upon (*a*) the power of the horse to respond, (*b*) the extent of contact, and (*c*) the time elapsing since the last contact. Only when this time interval was very long would a horse no longer have detectable circulating antitoxin, and so come within the scope of this paper. Such horses would exhibit a secondary response to a single injection of antigen, producing considerable titres within 10 days. The distribution of these antitoxic values would resemble that shown in Fig. 1A for horses with low normal antitoxic titres at the time of injection ; it will be noted that the scatter of values is relatively small, 75 per cent being between 2 and 20 units per ml.

In horses with no previous experience of infection, a single dose of antigen would be followed by a primary response. With the dose used in this experiment no antitoxin would be expected to develop for 2 to 3 weeks after injection, but

all except the very poor responders would have produced some antitoxin after 4 weeks. An injection of antigen into horses with some history of infection would produce a range of individual responses falling within the limits, just described, for animals with considerable previous contact and those with no contact prior to injection. In the course of these intermediate responses, varying antitoxic titres produced after 10 days would be expected to show considerable increases 28 days after injection.

Antitoxic responses 10 *days after injection.*

Fig. 1B shows the distribution of antitoxic values of 1000 horses 10 days after the initial injection of 5 ml. of A.P.T. ; none of these horses had detectable circulating antitoxin at the time of injection. It will be seen that nearly 60 per cent failed to respond, and that those which did respond formed two, or probably three populations. The largest of these corresponded to the single population in Fig. 1A (horses with low normal values at the time of injection), and constituted roughly 20 per cent of the total group shown in Fig. 1B. There is a suggestion

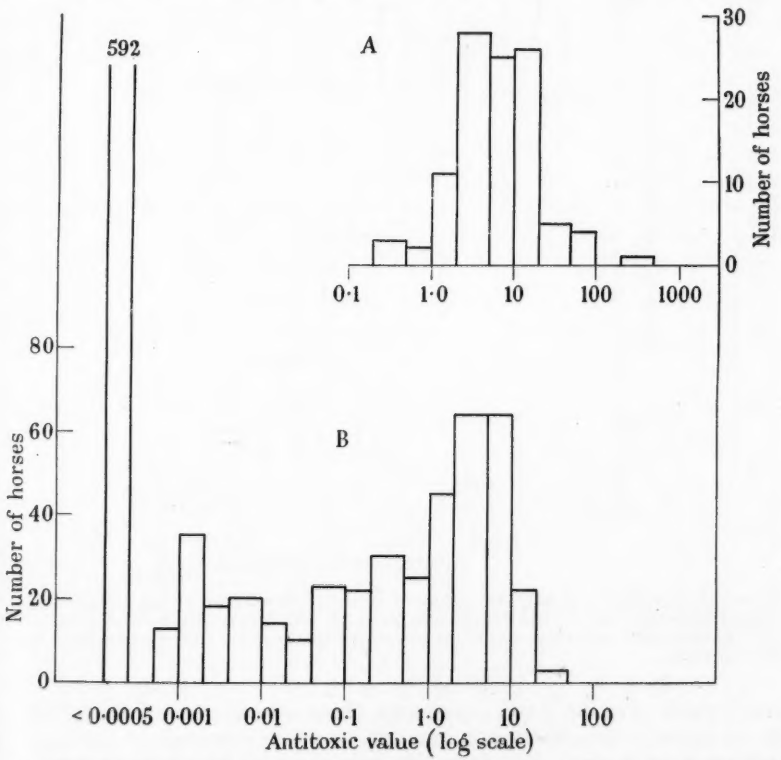

FIG. 1.—The distribution of antitoxic values of normal horses 10 days after an injection of 5 ml. of diphtheria A.P.T. A. 105 horses with 0·001 to 0·01 unit per ml. normal antitoxin. B. 1000 horses with less than 0·0005 unit per ml. normal antitoxin.

of a second population of slightly lower values between 0·04 and 0·5 unit per ml., and a definite population of low values round 0·001 unit. It is not surprising that as many as 20 per cent of the horses with no circulating antitoxin should exhibit fairly well-established basal immunity. The majority of these horses were aged, and had probably received some contact with infection in early life, but many years might well have elapsed without further contact owing to the decline in epidemics among the population.

Antitoxic response 28 days after injection.

Table II shows the antitoxic values of the same horses 28 days after injection, grouped according to the response at 10 days. Of the 592 horses which had produced no antitoxin 10 days after injection (Fig. 2c), all but 93 had responded

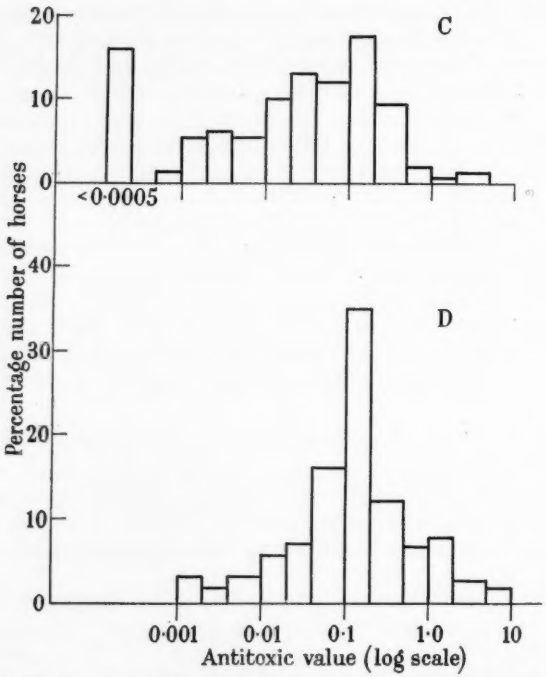

FIG. 2.—The distribution of antitoxic values of normal horses 28 days after an injection of 5 ml. of diphtheria A.P.T. (percentage numbers). C. 592 horses with no detectable antitoxin 10 days after injection. D. 133 horses with titres between 0·0005 and 0·04 10 days after injection.

to some extent after 28 days. Although these 93 animals might be regarded merely as initially completely non-immune, general experience in tracing curves of response suggests that they were also refractory to diphtheria immunization. The remaining 499 horses which had failed to respond 10 days after injection formed a distribution with a large scatter, showing a peak at 0·1 unit per ml.

TABLE II.—*Showing the Antitoxic Values of* 1000 *Horses with no Detectable Normal Antitoxin* 10 *and* 28 *days after an Injection of Diphtheria A.P.T.*

A.T. value 10 days after inj. (u./ml.).	Total number of horses.	Number of horses reaching values 28 days after injection (units/ml.).																Number changing from 10–28 days.		
		Under 0·0005	0·0005	0·001	0·002	0·004	0·01	0·02	0·04	0·1	0·2	0·5	1·0	2·0	5·0	10	20	−	0	+
<0·0005	592	93	8	32	34	32	60	76	71	105	57	12	5	7				—	93	499
0·0005	13					1	0	1	3	6	1	1						0	0	13
0·001	35			2	2	0	2	2	10	13	1	1	2					0	2	33
0·002	18						3	0	2	6	2	1	2	0	2			0	0	18
0·004	20					2	1	1	2	4	3	5	2					0	2	18
0·01	14			2	0	0	0	1	1	5	2	0	1	2				2	0	12
0·02	10					1	0	1	1	4	3							1	1	8
0·04	23						1	3	2	8	4	1	3	1				4	2	17
0·1	22						1	0	1	8	3	5	3	1				2	8	12
0·2	30								1	4	7	9	4	3	2			5	7	18
0·5	25								2	2	4	6	4	6	0	1		8	6	11
1·0	45									1	3	18	10	12	1			22	10	13
2·0	64											7	20	34	3			27	34	3
5·0	64											7	8	30	8	10	1	45	8	11
10	22										1	0	1	6	9	5		17	5	0
20	3														1	2		3	0	0
Total	1000	93	8	36	36	36	68	85	96	166	91	73	65	102	26	18	1	136	178	686

and a pronounced tail below this peak. It appears probable that all values below 0·01 unit 28 days after injection represented poor responses to primary stimulation, and the few high values were presumably intermediate responses reached by good immunizers with slight natural immunity before injection. It is suggested that many of the values at 0·1 unit per ml. were reached as a result of a slow intermediate response, but the large number of titres between this level and 0·01 unit suggest that some good responders to primary stimulation also contributed to this peak in the distribution.

Horses with 10-day values ranging from 0·0005 to 0·04 unit per ml. produced titres of comparable distributions after 28 days (Fig. 2D). The great differences between the 10-day values and the similarity between the 28-day values may be accounted for by slight differences in the times at which antitoxin-production started in the various animals. Such differences might in some cases amount to only one or two days, because a latent period of less than 8 days would not be expected prior to an intermediate response. Those horses in which antitoxin-production was not initiated until the 11th day (or later) after injection would appear in the top line in Table II ; this fact accounts for the existence of a peak at 0·1 unit in the distribution of 28-day values of both the non-responders and the horses with values between 0·0005 and 0·04 unit 10 days after injection. Whereas Fig. 2C shows an uneven distribution of more than one population, Fig. 2D shows a fair approximation to a normal distribution with a rather large scatter and a very marked peak at 0·1 unit. This suggests that the majority of animals in this group responded to injection in the same manner. Table II provides evidence in support of this conclusion ; nearly all these horses gave intermediate responses.

The remaining horses, with antitoxic titres of 0·1 unit per ml. or higher 10 days after injection, cannot logically be grouped together. Of those producing 0·1, 0·2 or 0·5 unit per ml. at 10 days, a considerable proportion rose appreciably in value 28 days after injection. It thus appears that a value as high as 0·5 unit 10 days after injection does not necessarily indicate that a typical secondary response has occurred. Owing to the wide levels of testing, however, some horses which appeared to have doubled in value between 10 and 28 days might only have increased by about 20 per cent. Such an increase could be expected in a secondary response if the value 10 days after injection did not represent the maximum height, and if the rate of fall after the peak was slight. It appears from Table II, however, that the lowest 10-day value representing a secondary response in the majority of animals is 1·0 unit per ml. ; most horses giving a lower value than this were intermediate responders.

The results clearly show the limitations of the conclusions which can be drawn from the value of a single blood sample taken either 10 or 28 days after an injection of antigen. For example, values of 0·1 unit per ml. were obtained 28 days after injection, from horses whose 10-day values ranged from under 0·0005 unit to 1·0 unit per ml., and to illustrate this point, hypothetical response curves have been constructed from some of the values given in Table II, and are shown in Fig. 3.

Curve 1 represents the response of a horse producing 0·5 unit per ml. 10 days after injection, and is typical of a relatively rapidly-falling secondary response. Curves 2 and 3 show the intermediate course followed by horses with 10-day values of 0·01 and 0·001 unit per ml. respectively, and Curves 4 and 5 depict

primary responses in horses failing to produce any measurable antitoxic titre 10 days after injection. All the horses shown in Fig. 3 had antitoxic values of 0·1 unit per ml. 28 days after injection.

These results suggest that when only one blood sample can be taken for the purpose of testing responses in a large-scale investigation, the most information can be obtained if it is taken 10 to 14 days after a single injection. So long as the dose given were small, an increase in titre would show the existence of some potential immunity, but the significance which could be attached to the titres reached would depend upon the dose given. The rapidity of development and magnitude of a response of the intermediate type would clearly depend upon the size of dose given ; if this were small, a fairly clear-cut division might be obtained

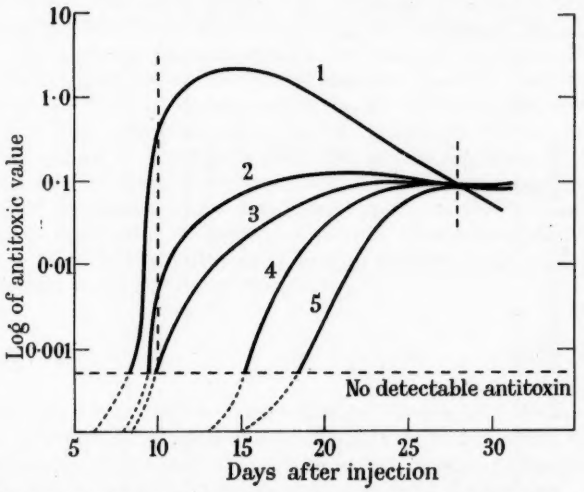

FIG. 3.—Hypothetical response curves constructed from values 10 and 28 days after injection given in Table II.

between the early titres reached in a secondary and in an intermediate response. On the other hand, initial rises in titre due to an intermediate response might be delayed, and some animals erroneously regarded as non-immune.

Tests upon a single blood sample taken 28 days after injection would give results which were very difficult to interpret. If the dose were relatively large, some measure of response would occur in all but the most refractory animals, and the type of response, over a large range of 28-day values, could not be estimated. If, on the other hand, the dose were small, an overlapping of values from rising intermediate and falling secondary responses would almost certainly occur. It is clear that whenever possible a minimum of two blood samples should be taken after injection, if a good idea of the degree of potential immunity and the type of response is to be obtained. These considerations should be borne in mind in the interpretation of values reached after injection of boosting doses in human prophylaxis.

Response to a second injection of A.P.T.

The majority but not all of the horses with no detectable antitoxin on arrival received a second dose of 5 ml. of A.P.T. 28 days after the first injection. Fig. 4 shows the antitoxic response of 850 horses to this second injection as judged by the titre of blood samples taken 14 days later.

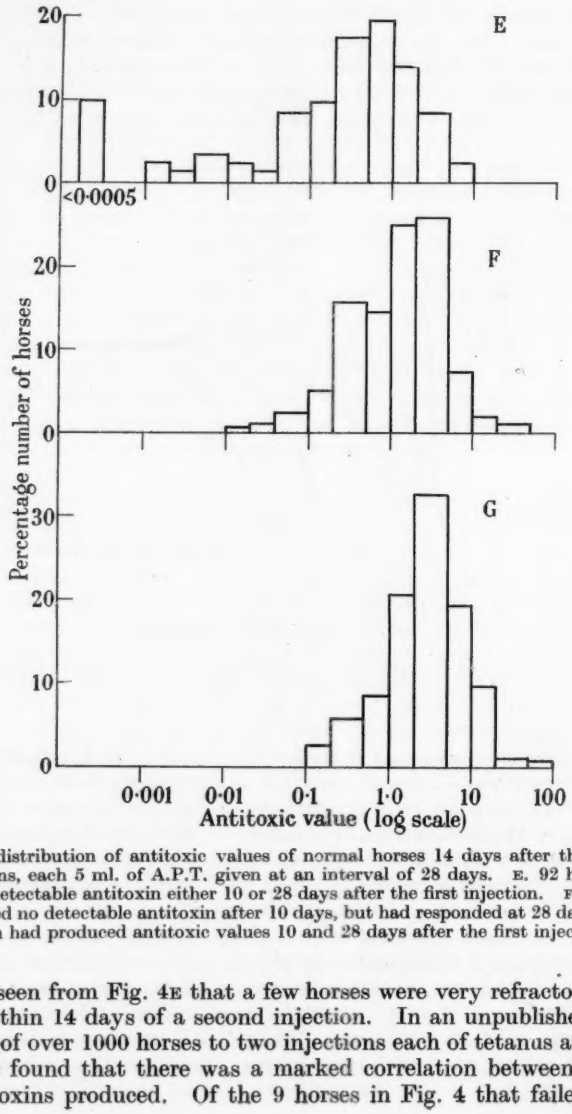

FIG. 4.—The distribution of antitoxic values of normal horses 14 days after the second of two injections, each 5 ml. of A.P.T. given at an interval of 28 days. E. 92 horses which showed no detectable antitoxin either 10 or 28 days after the first injection. F. 433 horses which showed no detectable antitoxin after 10 days, but had responded at 28 days. G. 325 horses which had produced antitoxic values 10 and 28 days after the first injection.

It will be seen from Fig. 4E that a few horses were very refractory, and failed to respond within 14 days of a second injection. In an unpublished analysis of the responses of over 1000 horses to two injections each of tetanus and diphtheria A.P.T. it was found that there was a marked correlation between the titres of the two antitoxins produced. Of the 9 horses in Fig. 4 that failed to produce

any measurable diphtheria antitoxin after two injections of A.P.T., 5 showed an extremely poor response to tetanus prophylaxis, 2 were poor, another definitely below the average, and only one recorded an average response. It is therefore very probable that these horses were generally refractory to immunization with toxoids. The peak of the main distribution in Fig. 4E occurs round 0·5 units per ml., but there is another small population of extremely low values peaking at 0·004 unit per ml., and the division between these two populations of values is clear-cut.

Fig. 4F gives the distribution of values of horses which had failed to respond 10 days after the first injection, but had produced antitoxin 28 days after, and shows a smaller scatter of values and a higher main peak than that shown by the non-responders to a single injection. There is a pronounced tailing-off immediately below the peak, suggesting a second population corresponding to the main distribution in Fig. 4E, and overlapping with the slightly higher value population. This group of horses was almost certainly composed of some animals with no potential immunity at the time of the first injection, and others with some natural immunity that produced antitoxin as a somewhat slow intermediate response to the first injection. This fact may account for the existence of two populations of values in this group after the second injection of A.P.T., the one corresponding to the main distribution of the non-immunes (Fig. E), and the other to that of the horses which responded rapidly to the first injection (Fig. G). These latter, 14 days after the second injection, produced a single population of values with a relatively small scatter and a marked peak at 2 units per ml.; the values appear to be normally distributed.

DISCUSSION.

The results shown in Fig. 4 suggest that two doses, each 5 ml. of A.P.T., were sufficient to establish good basal immunity only in those horses which had some potential immunity before injection, and possibly in some good responders among the originally non-immune. It is interesting to note the points at which peaking of various distributions occurs, and a comparison of Fig. 1A with Fig. 4G shows that a distribution of comparable range and peak value may occur after one and after two similar injections of antigen. Thus a third injection given to the horses of Fig. 4F would be expected to reduce the scatter of values below the main peak of the distribution, and produce an effect similar to that shown in Fig. 4G for the horses which were potentially immune before injection. A third injection would be expected to have the same effect on many, but not all of the originally non-immune animals in Fig. 4E.

The term " basal immunity " could with advantage be reserved for an adequate standard of immunological preparation of an animal for a certain purpose. Thus in work on horses, a standard is required such that an intensive course of hyper-immunization will result in the production of high value antitoxin of good avidity. If basal immunity is not sufficiently established, lower value antitoxin of poor avidity may be produced as a result of hyperimmunization (Barr and Glenny, 1945). Satisfactory basal immunity must at all times include the power to set in motion a very rapid secondary response to an injection of antigen or to toxin produced in the body as a result of infection. This immunological condition is

therefore one of high potential immunity ; the latter term implies any power of response superior to that of a completely normal animal.

The exact nature of the various types of response has not been clearly defined since publication of the original work of Glenny and Sudmersen (1921). When very large doses of prophylactic are used, the accepted classification into the primary, intermediate and secondary types breaks down. A primary response is essentially the response of a completely non-immune animal to the first dose of antigen, but the time at which antitoxin first appears in the circulation and the height of the response depend upon the dose given. If this is very large the response curve may resemble that of the secondary type, and the original conception of a latent period of 3 to 4 weeks does not hold. A primary response may be given by an animal which has had some contact with infection, but has lost the potential immunity developed thereby and relapsed in consequence to the " normal " state. Secondary responses are distinguished by the shorter latent period, which seldom exceeds 7 days and may be 4 to 5 days ; the height is normally reached 10 to 15 days after injection, and is almost independent of the dose given. The peak of a secondary response is not usually maintained for more than a few days, but the rate of loss varies enormously with the type of antigen used and the amount of earlier natural or artificial stimulation experienced by the animal. It sometimes happens that an animal which has previously given a typical secondary response, and does so again at some later stage, shows an abnormal type characterized by a slower rise and a peak value reached as late as 21 days after injection. An intermediate response can only be given by an animal which has some degree of potential immunity, and thus differs essentially from a primary response. In the absence of an exact knowledge of the previous immunological history of an animal no clear-cut line of demarcation can be drawn, however, between a primary and a weak intermediate response. At the other end of the scale no distinction can be made between a good intermediate and a weak secondary response.

It is possible that two stages are always necessary in the development of antitoxin, some antigen producing or increasing potential immunity, and the remainder acting upon already sensitized cells or their products to produce antitoxin. Such a process would account for the slow appearance of antitoxin following primary stimulation, if some of the injected antigen were still available by the time that sufficient sensitization of antitoxin-producing cells had been achieved. In secondary stimulation the antigen may also play a dual role, the bulk of injected material acting upon the sensitized cells, but a portion increasing the degree of potential immunity by acting upon new tissue. If this were the case, although a small dose given as a secondary stimulus will produce almost as high a titre as a large one, a large dose may have the advantage of greatly increasing potential immunity.

The existence of such a variety of responses in a large number of animals with some degree of potential immunity and no circulating antitoxin may assist to some extent the consideration of the processes involved in the development of active immunity. Of more immediate concern, however, is the application of these findings to human prophylaxis against diphtheria. Great differences in degree of potential immunity may exist among older schoolchildren and adults who have received a basic course of immunization in very early life. The results reported in this paper suggest that in the event of an epidemic, some persons who

had had no prophylactic injections since infancy might require at least two boosting doses before they could be regarded as adequately protected against infection.

SUMMARY.

A thousand normal horses with less than 0·0005 unit/ml. of circulating diphtheria antitoxin were given an injection of A.P.T., and blood samples tested 10 and 28 days later; 850 horses receiving two such injections had an additional test 14 days after the second injection. Just over 40 per cent appeared to have some potential immunity because they produced some antitoxin within 10 days of the first injection.

Very great differences existed in the degree of potential immunity and capacity to respond to injection. Whereas most horses with titres of 1 unit/ml. or more 10 days after injection appeared to produce a secondary response with a fall in value at 28 days, the majority of those with lower 10-day values showed a considerable subsequent rise. This latter sequence is taken as evidence of intermediate response.

The degree of potential immunity existing in animals at the time of an injection of A.P.T. cannot be judged by the magnitude of the antitoxic response measured either 10 or 28 days later. Tests on at least two blood samples are necessary; the relation between the values at 10 and 28 days would depend upon the dose given, in relation to the pre-existing degree of potential immunity. These facts should be taken into account in the interpretation of antitoxic responses to boosting doses in human prophylaxis.

Though differences in response are reduced after a second injection of A.P.T., further doses would be needed in some animals to establish sufficient basal immunity to ensure a secondary response.

Great differences in degree of potential immunity may exist among older schoolchildren and adults who have received no prophylactic injections since infancy. Thus in the event of an epidemic, some such persons might require two or more boosting doses to establish adequate protection against infection.

The large variety of responses exhibited may assist to some extent the consideration of the physiological processes involved in the development of active immunity.

I am indebted to Mr. A. T. Glenny, F.R.S., for helpful criticism, and for making available the earlier figures on normal antitoxic values.

REFERENCES.

Barr, Mollie, and Glenny, A. T.—(1945) *J. Hyg., Camb.*, **44**, 135.
Glenny, A. T., and Sudmersen, H. J.—(1921) *Ibid.*, **20**, 176.

THE SYNERGISTIC ACTION OF THE ANTERIOR AND POSTERIOR PITUITARY HORMONES AND THEIR POSSIBLE RELATION TO ECLAMPSIA.

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THE hypertension in toxaemia of pregnancy is obviously of functional nature. There is a rapid decline of the blood pressure during convalescence ; the disease is most commonly seen in primiparous patients, and rarely affects these individuals during subsequent pregnancies. Concomitant with the fall in blood pressure there is a marked diuresis, and albumen disappears from the urine. Cohnheim (1880) was apparently the first to regard hypertension as the cause of the symptoms of eclampsia, and he believed that albuminuria was due to spasm of the renal arteries. Volhard (1918) suggested that eclampsia was a disease of all the small terminal arterioles, and a similar view was held by Hinselmann (1924) and Fahr (1924). Nevermann (1922) and Hinselmann (1924) found direct evidence of arteriolar spasm when studying the vessels of the nail-bed in toxaemic patients. Similar results were obtained by Mylius (1928) and Wagener (1933) on examination of the retinal vessels, and Bell (1932) demonstrated gross thickening of the capillary basement membranes as well as other vascular changes in fatal cases of eclampsia.

The mechanism which maintains this functional hypertension may be humoral or nervous. The search for a humoral agent has been made on many occasions with varying success. A substance resembling extract of posterior pituitary was found by Anselmino and Hoffmann (1931*a*, *b*, *c*) in the ultra filtrates of blood from eclamptic and pre-eclamptic patients. Coester (1935) and Teel and Reid (1939) demonstrated an anti-diuretic factor in the urine of toxaemic patients. This statement, however, has been contradicted by Byrom and Wilson (1934), Theobald (1934) and Levitt (1936). Similarly, the claim made by Anselmino and Hoffmann (1931) to have demonstrated a pressor substance in the blood of toxaemic patients has been challenged by de Wesselow and Griffiths (1934) and Hurwitz and Bullock (1935). More recently Mukherjee (1941) has demonstrated a pressor substance in the blood of severely toxaemic patients by methods similar to those of Anselmino and Hoffmann. Ham (1941) claims to have found an anti-diuretic substance in the urine of such cases which differs in certain respects from pituitrin. Although there is thus some controversy regarding the part played by " pituitrin " in the onset of toxaemia, there is little doubt that toxaemic patients are more sensitive to pituitrin than normal pregnant women. This has been demonstrated repeatedly (Dieckmann and Michel, 1937 ; Schockaert and Lambillon, 1937 ; de Valera and Kellar, 1938).

Experimentally the results of pituitrin administration in animals have been very variable. Fauvet (1931) and Végh and von Pallos (1937) claimed to have produced the pathological lesions of eclampsia by this means, but the doses of pituitrin used were extremely large, and well beyond the amounts which might be expected in the natural disease. Byrom (1938), using smaller doses, could find no definite lesions in experimental animals, but he demonstrated that gonadal hormones sensitized an animal to the action of pituitrin. Browne (1946) demonstrated a similar sensitizing action on the part of synapoidin, a preparation containing gonadotrophins of placental and pituitary origin. This work of Browne followed upon that of Smith and Smith (1939), who found an abnormally high concentration of gonadotrophins in the serum of eclamptic and severe pre-eclamptic patients. They also found (Smith and Smith, 1940) a decrease of progesterone and oestrogen.

From the foregoing it seems possible that the hypertension of pre-eclampsia might be due to normal amounts of pituitrin acting upon a vascular system sensitized by gonadotrophins. However, it remains to be seen whether lesions resembling those of eclampsia can be produced by a hypertensive agent such as pituitrin, and whether gonadotrophins modify the action of pituitrin in any way. We have investigated these experimental problems, and the following is a report of our results up to the present.

METHODS.

The animals used were normal adult rabbits weighing approximately 2·0 kg. They were housed in simple urine cages and given an ordinary mixed diet of bran, oats and greens. Urine was collected daily for analysis.

Histological.

After autopsy, pieces of material were selected from the various organs. Formol-saline and alcoholic picric acid were used as fixatives. Paraffin sections of the formol-fixed material were stained by haematoxylin and eosin, Foot's method for reticulum and Weigert's method for fibrin. Material fixed in picric acid was stained by Bauer-Feulgen's method for glycogen.

SERIES I.—*Five Female Rabbits given 1/2 ml. Pituitrin in Olive Oil Subcutaneously Twice Daily.*

During the experimental period all animals tended to lose condition. For the first three days their appetite diminished and they lost weight. On the 5th day one of the animals appeared to be ill and sat hunched up in the corner of the cage. It was killed by a blow on the back of the neck. The remaining animals appeared to be healthy and their appetite returned. Another two animals were killed on the 7th day ; a fourth animal was sacrificed on the 21st day, and the last on the 31st day. During the first few days there was some oliguria, but this was probably related to their diminished intake of food and not to any renal dysfunction.

At post-mortem naked-eye changes were found in the liver of the animal which was killed on the 5th day, and in that of one of the animals killed on the 7th day. The changes consisted of irregular patches of pallor on the upper surface mainly of the right lobe. This pallor appeared to be entirely superficial,

and a cut made through the pale patches showed a normal underlying parenchyma. Over the remainder of the surface the lobular markings were prominent.

In the case of the other animals no change apart from an increased prominence of the lobules could be seen in the livers. There was some degree of pallor of the renal cortex in all animals.

Histology.

Liver.—The liver in the first three animals presented a rather similar appearance. There was well-marked pallor of the centro-lobular cells. Closer examination showed that the cells were swollen and globular with hydropic cytoplasm. A few were vacuolated. Many cells were bi-nucleated, but in a number the nuclei had disappeared. The sinusoids were almost obliterated in parts by the swollen cells. Numerous brownish-black pigment granules were present in these cells.

In the remaining two animals there were tiny infrequent areas of midzonal necrosis. These appeared to be in a healing stage and many of the necrotic cells had disappeared. Those still visible were shrunken, opaque and very eosinophilic. Nuclei had disappeared. The area was infiltrated by macrophages, lymphocytes and a few polymorphs.

Kidneys.—The renal changes were very similar in all cases. In general the outer cortical glomeruli appeared to be somewhat smaller than normal, whereas those in the juxta-medullary region were much enlarged and resembled human glomeruli. There was dilatation of the cortical veins. The glomerular capillaries varied in diameter and there was an irregular congestion. A very occasional loop appeared to be necrotic and infarcted. In a few of the proximal convoluted tubules the epithelial nuclei were pyknotic, but in general they were healthy. Many of the distal tubules were dilated and some contained albuminoid casts.

SERIES 2.—*Seven Male Rabbits receiving Pituitrin in Oil Subcutaneously.*

These animals were divided into two groups. Group A consisted of 4 animals which were castrated 2 weeks before the experiments began. One animal in Group A received 1/2 ml. of pituitrin in oil twice daily for 20 days; another animal was given 5 ml. of pituitrin in oil daily for 8 days, and the remaining two were given 5 ml. of pituitrin in oil daily for 21 days.

The three animals of Group B were normal males. Each received 5 ml. of pituitrin in oil daily for 10 days. The animals of both groups became somewhat apathetic and lost a little weight, but showed no other symptoms. There was no sign of oliguria. They were killed by a blow on the back of the neck 4 hours after the last injection.

At post-mortem no change could be seen in the livers of 3 animals in Group A and 1 animal of Group B. In one of the rabbits in Group A receiving 5 ml. of pituitrin over a period of 21 days and in 2 animals of Group B there were large irregular areas of pallor on the upper surface of the right hepatic lobe similar to those seen in animals of Series 1. As before, the pallor was quite superficial. It was noted that in these animals the vessels of the ligamentum teres were congested.

The renal cortices of all the animals in both groups were paler than normal.

Histology.

Liver.—Minimal changes were found in 2 animals of Group A, namely one receiving 1/2 ml. pituitrin for 20 days, and one receiving 5 ml. pituitrin for 8 days, and in one animal of Group B. These consisted of a hydropic degeneration of the cells of the centrolobular zones. The cells were swollen, and the cytoplasm appeared to be flocculated into coarse clumps. Nuclei were mainly normal, but a few appeared to be undergoing karyolysis. Individual cells were seen in all parts with basophilic cytoplasm and dark nuclei; a few of these were binucleated and the appearances suggest regeneration. These changes were most marked in the Group B animal.

Similar changes were found in the remaining animals, but in addition there were fairly large but very infrequent areas of necrosis (Fig. 1 and 2). These areas were almost entirely confined to the parenchyma near the free edge of the liver. They were of an irregular ellipsoid shape affecting the midzone on one aspect of a lobule. The necrosis was advanced, and the cell columns were fused into a homogeneous pink mass in which a few patent sinusoids could still be seen. Nuclei had almost entirely disappeared. At the edge of the necrotic area there were occasionally a few small multi-nucleated cells containing dark brown pigment. Gram's stain showed a deposit of fibrin in some of the sinusoids. The portal vein radicles were dilated, and very occasionally the related hepatic arteriole was necrotic. A marked polymorph reaction was present in the sinusoids surrounding the necrotic area. Glycogen was much reduced, and was found mainly around the centrolobular veins.

Kidney.—The changes in this organ were very similar in all animals, but they were perhaps less marked in the 3 animals showing minimal hepatic changes. As in the previous series, the glomeruli of the juxta-medullary region were somewhat enlarged. In the outer cortical glomeruli there was irregular dilatation and congestion of the capillaries. The epithelium of the first convoluted tubules was swollen and finely vacuolated. The more distal tubules were dilated and occasional haemoglobin and eosinophilic casts were present.

In the animals showing more marked hepatic changes there was apparent infarction of one or two capillary loops in an occasional glomerulus. Many of the capillaries of the outer cortical glomeruli appeared to be occluded by swelling of their walls, and in some instances there seemed to be reduplication of the basement membrane. The tubular changes were much the same as before, but eosinophilic casts were more common.

SERIES 3.—*Four Adult Female Rabbits receiving* 500 *I.U. of Chorionic Gonadotrophin* (*Gonan, B.D.H.*) *Subcutaneously Twice Daily.*

Animal 1 received 16 injections, Animal 2, 19 injections, and Animals 3 and 4, 21 injections. Throughout the whole experimental period the animals remained healthy. There was no loss of appetite or weight, and there was no diminution in the output of urine. Four hours after the last injection the animals were killed by a blow on the back of the neck.

At post-mortem no gross change could be found apart from the obviously active ovaries, which were enlarged and contained many corpora lutea in various stages of development. The uterine horns were enlarged and congested.

Histology.

Liver.—No marked change could be seen in this organ. In one or two lobules of one liver, however, there were occasional cords of narrow cells with basophilic cytoplasm and hyperchromatic nuclei.

Kidney.—All kidneys appeared to be completely normal.

SERIES 4.—*Seven Rabbits receiving Chorionic Gonadotrophin plus Pituitrin.*

Six of these animals were adult females, and the seventh a castrated adult male. All received 500 International Units of chorionic gonadotrophin (Gonan, B.D.H.) twice daily for 2 days, followed by the same amount of chorionic gonadotrophin plus pituitrin 1/2 ml. in oil twice daily. The male animal developed a skin infection on the 13th day, and was killed before it became severe. It appeared to be otherwise healthy. Four of the female rabbits were killed on the 19th day, and 2 on the 21st. The female animals behaved in a manner similar to those of Series 1 receiving pituitrin alone. They became apathetic and lost their appetite. There was some loss of weight and oliguria, probably as a result of the diminished intake of food.

At post-mortem no gross change could be seen in the major organs. The lobular markings of the livers appeared to be more prominent than usual, and the renal cortices were pale. No sign of haemorrhage or necrosis could be detected with the naked eye. There was marked luteinization of the ovaries of the 6 female animals. In the male animal both liver and kidneys were somewhat paler than normal.

EXPLANATION OF PLATES.

FIG. 1.—Series 2. Liver from animal receiving 50 units of pituitrin in oil daily. There is marked hydropic change in the liver cells. Haematoxylin and eosin. × 105.

FIG. 2.—Series 2. Liver from animal receiving 50 units of pituitrin in oil daily. There is advanced midzonal and centrolobular necrosis. Haematoxylin and eosin. × 105.

FIG. 3.—Series 6. Liver from animal in Group B receiving anterior pituitary extract plus 2·5 units of pituitrin in oil twice daily. Note the small rounded areas of necrosis and cellular reaction near the portal tract. Haematoxylin and eosin. × 105.

FIG. 4.—Series 6. Liver showing small area of periportal necrosis. Note the triangular plan of the necrotic area. Haematoxylin and eosin. × 105.

FIG. 5.—High-power view of the portal tract in Fig. 4. The hepatic arteriole is greatly distended and necrotic. Haematoxylin and eosin. × 500.

FIG. 6.—High-power view of hepatic arteriole from human case of eclampsia for comparison with Fig. 5. Haematoxylin and eosin. × 500.

FIG. 7.—Series 6. Renal glomerulus showing infarction of a lobule. Haematoxylin and eosin. × 500.

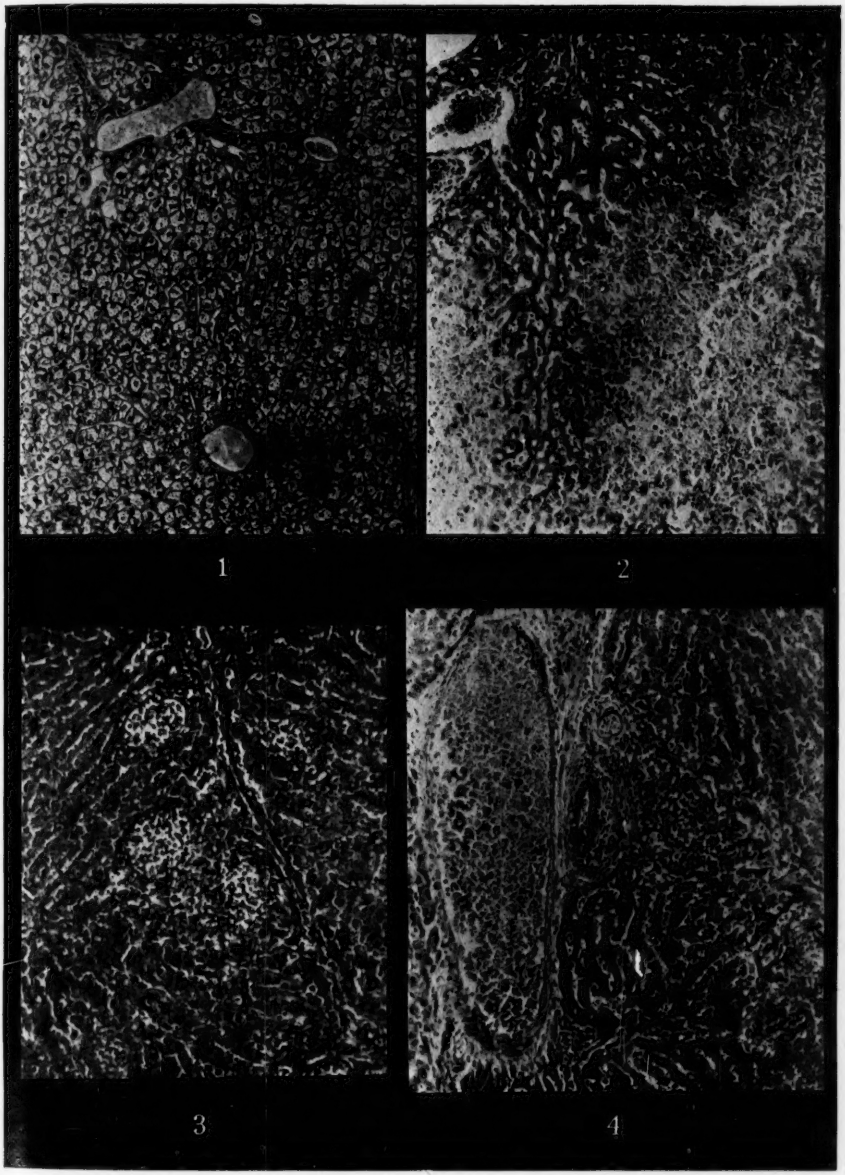
FIG. 8.—Series 6. Renal glomerulus showing hyaline thrombus material applied to walls of capillaries. Some capillaries are occluded, others narrowed. Haematoxylin and eosin. × 500.

FIG. 9.—Series 8. Liver from animal receiving urine from toxaemic patient plus 2·5 units of pituitrin in oil daily. There are zones of necrosis in the periportal and centrolobular areas. Haematoxylin and eosin. × 105.

FIG. 10.—Series 8. Liver showing periportal necrosis. Note the triangular plan of the lesion. Haematoxylin and eosin. × 105.

FIG. 11.—Series 8. Kidney showing eosinophilic casts in the ascending limbs of Henlé, and vacuolation of the proximal tubules. Haematoxylin and eosin. × 105.

FIG. 12.—Series 8. Kidney showing complete necrosi of glomerular capillaries and afferent arterioles. Haematoxylin and eosin. × 500.



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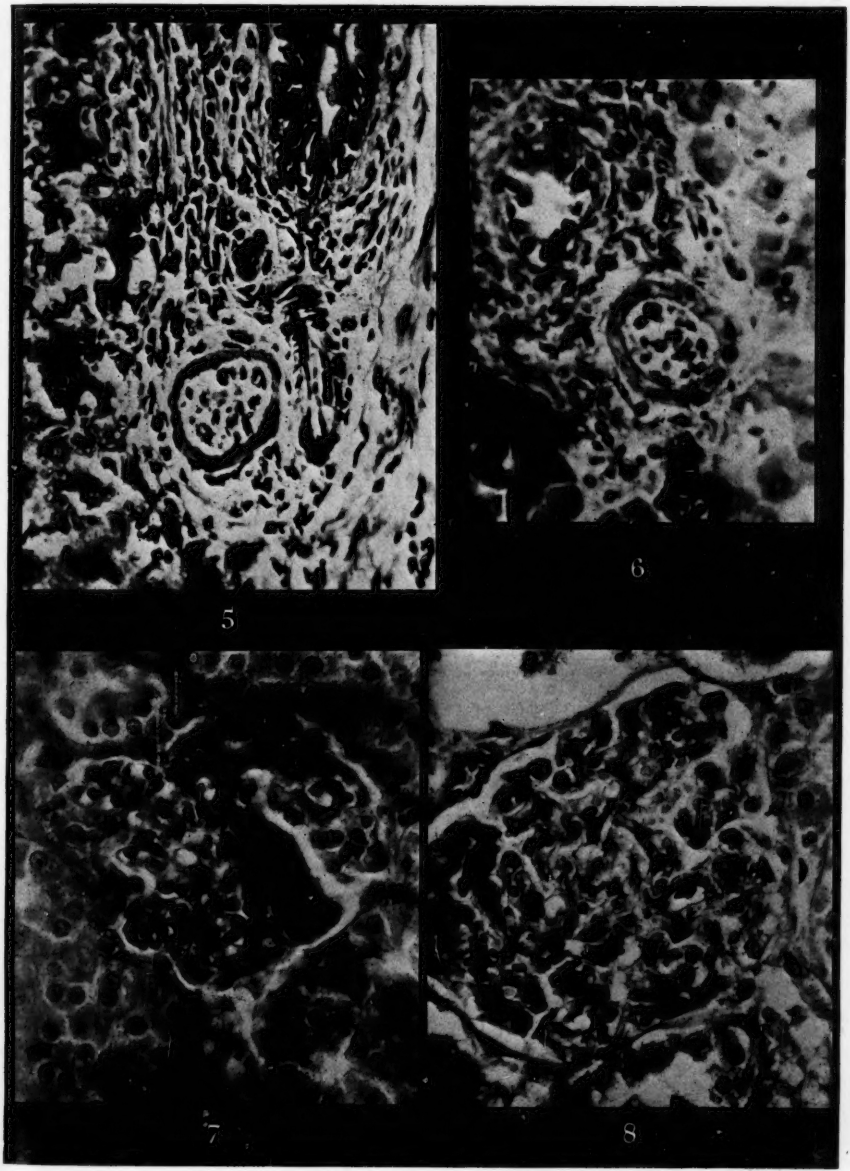

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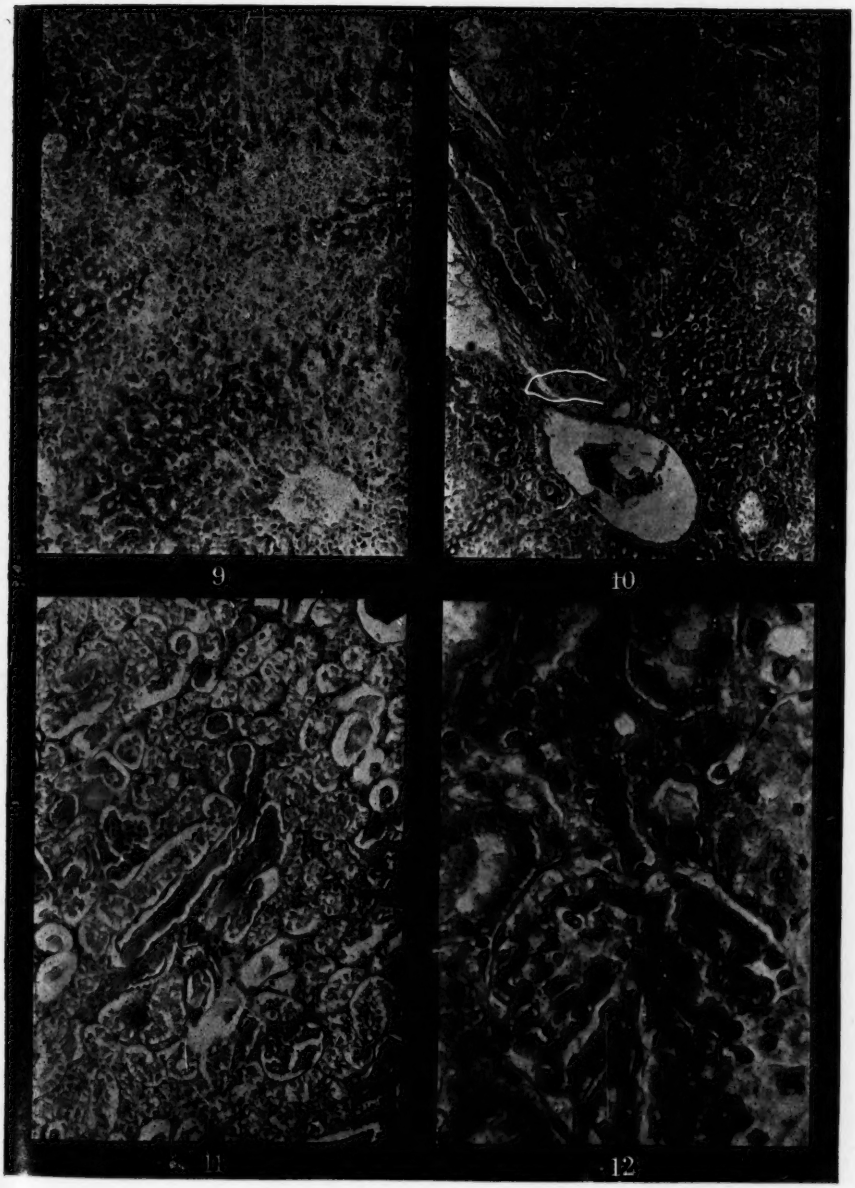

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Histology.

Liver.—In the liver of one of the female rabbits killed on the 21st day there were one or two areas of necrosis similar to those seen in Series 2. The necrosis was of the same coagulative type and occurred in the midzone. The liver of one other female rabbit, receiving 19 injections, showed hydropic degeneration of the central zones. No change could be seen in any of the other livers. Glycogen was much diminished and patchy in distribution.

Kidney.—The changes were similar to those seen in Series 2, but were not so marked.

SERIES 5.—*Animals receiving Sheep Anterior Pituitary Lobe Extract.*

Six adult female rabbits received 2 ml. of the fresh alkaline extract of sheep anterior pituitary lobe twice daily by intravenous injection. Two were killed on the 12th day, 2 on the 19th day and 2 on the 20th day. Throughout the experiment the animals were healthy and continued to take their food.

Preparation of the pituitary extract.

Fresh sheep pituitaries, collected immediately after the death of the animals, were used for this purpose. As soon as possible after collection the anterior lobes of these glands were dissected off and ground up with silver sand. The paste was then extracted for 30 minutes with 0·05 N caustic soda. Thereafter the fluid was decanted and centrifuged for 10 minutes and filtered to remove all particulate matter. The resulting fluid was such that 2 ml. were approximately equivalent to the anterior lobes of two pituitary glands.

At post-mortem only one animal showed any notable changes. In this animal there was some congestion of all the abdominal organs. The ovaries of all animals contained mature follicles and a few haemorrhagic corpora lutea. There was in addition congestion and some enlargement of the uterine horns.

Histology.

Liver.—There were some fatty changes in the central zones of the liver of the animal, which showed congestion of the abdominal organs. No abnormality could be found in any of the other animals apart from diminution of glycogen.

Kidney.—In all animals there appeared to be some congestion of the kidneys, but otherwise these organs were normal.

SERIES 6.—*Ten Animals receiving Pituitrin plus Extract of Sheep Anterior Pituitary.*

Ten adult female rabbits were used. They each received 2 ml. by injection, of the same anterior pituitary extract, previously described, twice daily for 2 days. On the 3rd day they were divided into two groups. Group A, consisting of 4 animals, were given 50 units of pituitrin intraperitoneally. All animals died, death occurring in the following order:

R_1—2 hours after injection of pituitrin.
R_2—$2\frac{1}{2}$,, ,, ,,
R_3—10 ,, ,, ,,
R_4—$5\frac{1}{4}$,, ,, ,,

All animals in this group died in a state of spasticity, with necks retracted and limbs somewhat extended. Mechanical stimuli evoked a generalized tremor, most marked in the muscles of the neck and back. Cyanosis was prominent. The general post-mortem features were similar in all animals. The liver showed areas of extreme subcapsular congestion almost amounting to haemorrhage. Marked peri-lobular congestion was present on the cut surface. There was marked pallor of the outer cortical zone of the kidneys, with considerable congestion of the medulla. The meninges were very congested.

Group B, 6 animals, continued to receive 2 ml. of anterior pituitary extract twice daily, but in addition each animal was given 2·5 units of pituitrin in oil by injection twice daily. The outcome in this series was as follows :

R_5	lived for	91	hours.
R_6	,,	190	,,
R_7	,,	106	,,
R_8	,,	158	,,
R_9	,,	64	,,
R_{10}	,,	206	,,

After the first day all animals became apathetic. Their appetites deteriorated and the urinary output diminished considerably. In R_5 there was complete anuria from the second day of the pituitrin injections. Anuria developed on the following day in R_7. In all other animals the daily output never exceeded 18 ml. The urine was loaded with albumen. Before death they became cyanosed and drowsy, and eventually lapsed into coma. They responded in a jerky reflex manner to mechanical stimulation.

At post-mortem the changes were very similar in all animals. The pericardial and pleural cavities contained abundant free fluid, and there was an excess in the peritoneal cavity of Animals 5, 7, 8 and 9. Small areas of haemorrhage were present on the epicardial surface of the heart, and the pleural surfaces showed numerous areas of haemorrhage varying in size from a pin-point to one-quarter of a centimetre in diameter. Areas of pale necrosis alternating with others of extreme congestion were present on the surface of the liver. A few small areas of subcapsular haemorrhage were also noticed. The kidneys of Animals 5 and 7 showed small opaque yellow areas suggesting necrosis in the cortex. The medulla in these animals was congested and showed haemorrhages. In other animals there was cortical pallor. In Animal 6 the bladder contained blood.

Histology.

Despite the variation in dose of pituitrin these two groups did not show much variation in the type of lesion observed, but there was a difference in degree, the histological changes being more advanced in Group B.

Liver.—In five of the animals in Group B there was widespread hepatic necrosis affecting almost every lobule. Necrosis was also present in the remaining animal of Group B and in Animals 2, 3 and 4 of Group A, but it was not so marked. The changes were typified by the appearances found in one animal which died on the second day. The lesions, which were found in every lobule, were rather small and somewhat rounded. They occurred in all situations, central, midzonal, paraportal and peri-portal, but were most common in the last situation (Fig. 3). The

necrosis was of a coagulative type, the cells staining a homogeneous opaque pink with eosin and fusing to form solid columns. Nuclei in most instances had disappeared. In a few lobules the necrotic hepatic cells had disappeared and the area was filled by a mixture of polymorphs and mononuclears. In other lobules the sinusoids surrounding the areas of necrosis were congested and contained many polymorphs. In a few instances there were small areas of haemorrhage. Thrombosis of the sinusoids was quite common. Thrombosis was also noted in some of the portal venules and an occasional central vein. Many of the hepatic arterioles were necrotic (Fig. 4 and 5).

Kidney.—Gross lesions were present in 6 animals. The changes were most marked in the glomeruli. Many of the tufts showed infarction of one or more lobules, and in a few of them the whole tuft was affected (Fig. 7). The tufts were much enlarged and filled their capsules. The capillaries of most were filled by broad strands of hyaline pink material, giving a faint reaction with Gram's stain (Fig. 8). In other tufts the capillaries were greatly engorged, and the red cells appeared to have fused to form a homogeneous pink-staining mass. Many of the capillary walls were necrotic. In non-infarcted lobules of the tufts thrombus material could be seen applied to walls of individual capillaries. Some glomeruli showed an increased cellularity, while in the infarcted type cells were disappearing. In a few tufts there were occasional mitoses among the endothelial cells. A number of the afferent arterioles were thrombosed, and some were obviously necrotic. Thrombosis had also occurred in a number of veins, and this was accompanied by a slight leucocytic reaction.

Tubular changes were not marked in these animals. The second convoluted tubules and ascending limbs of Henlë were dilated, and a number contained hyaline casts. A few haemoglobin casts were also present. The epithelium of these tubules was somewhat flattened and the nuclei were commonly pyknotic. The epithelium of the first convoluted tubules was swollen and slightly vacuolated, but otherwise normal.

The kidneys of the other animals showed irregular dilatation of the glomerular capillaries with infarction of an occasional lobule. The changes, however, were not gross as in the other animals.

SERIES 7.—*Animals receiving Pituitrin plus Extract of Sheep Anterior Pituitary containing no Gonadotrophic Hormone.*

Three adult female animals were used. They each received 2 ml. of the anterior pituitary extract daily for 2 days, and on subsequent days 1/2 ml. of pituitrin in oil twice daily in addition. At the end of 10 days they were killed. Throughout the experiment they remained healthy. There was no oliguria or albuminuria.

Anterior pituitary extract.

This was made in exactly the same way as before, but on testing it was found that it failed to stimulate the ovaries of a rabbit.

At post-mortem the liver showed a few pale areas on the upper surface similar to those found in Series 1 and 2. There was no sign of haemorrhage. The renal cortices were relatively pale.

Histology.

Liver.—Apart from a little pallor of the cells in the central zones of the lobules no gross change could be found. There was no sign of necrosis in any part examined.

Kidney.—One or two glomeruli were congested, but no organic change could be seen in any part. The distal tubules were slightly dilated, but otherwise normal. There were no casts.

SERIES 8.—*Animals receiving Injections of Urine from Pregnant Patients plus Pituitrin.*

Early in our studies of toxaemia it was discovered that patients could be divided into two groups according to the concentration of gonadotrophins in their serum. In one group of toxaemic individuals the concentration was normal, while in the second it was greatly increased. In some cases of the second group there was also a high concentration of gonadotrophins in the urine. In the present experiment we made use of urine from these cases.

Fourteen adult rabbits were used. These were divided into four groups of 3, 3, 3 and 5 respectively. The first Group (A) consisted of 3 adult female rabbits. These animals received 15 ml. of urine intravenously from a normal pregnant woman at term daily for 14 days. The urine was an early morning catheter specimen and therefore concentrated. Group B, 3 adult female rabbits, were treated in the same manner, but the urine was obtained from a severely toxaemic patient. Group C, 3 adult female rabbits, were injected with 15 ml. of urine from a normal pregnant woman at term daily for 14 days. In addition each received 0·25 ml. of pituitrin in oil subcutaneously. Group D, 4 adult female rabbits and 1 castrated male, each received 15 ml. daily by intravenous injection of a fresh early morning catheter specimen of urine from a patient suffering from severe pre-eclampsia. At the same time each was given 0·25 ml. of pituitrin in oil subcutaneously. The animals of Group A, B and C remained healthy throughout the experimental period, apart from some loss of appetite. They were killed by a blow on the back of the neck on the 15th day. No gross abnormality could be found at post-mortem. All the animals of Group D died, the females at intervals of 5, 6, 10 and 13 days respectively. The male animal succumbed on the 7th day.

Every animal lost condition progressively. They became listless, with no appetite, and for 2 or 3 days before death they were definitely ill, sitting hunched up in the corner of the cage with "staring" coat and refusing all food. Oliguria was marked, and such urine as was passed was loaded with albumen. During the last day they became drowsy, eventually falling into a coma ending in death.

The female animal which died on the 5th day appeared to be shocked following the last injection of urine and pituitrin. It lay limp in a corner of the cage and was cold to the touch. Four hours later it died.

At post-mortem the most marked changes were found in the animals which survived longest. There were areas of subcapsular haemorrhage on the upper surface of the liver in all animals, with marked congestion of rhe vessels in the ligamentum teres and on the under-surface of the diaphragm. The hepatic parenchyma of the male animal and that of the two female animals dying on the 5th and 6th days respectively showed a general pallor, but no other gross change

could be seen. On the other hand there were small but definite areas of yellow opaque necrosis in the livers of the animals which survived until the 10th and 13th days. Similarly there were gross changes in the kidneys of the animals which survived longest. There was marked pallor of the renal cortex in all animals, but in these two the pallor was of a yellow, opaque type,and here and there tiny streaks of apparent haemorrhage could be seen. The medullae were congested, almost bloodshot, and again lines of apparent haemorrhage could be seen.

In the two animals which survived longest there was free fluid in the abdomen.

Histology.

Liver.—Apart from some slight pallor and swelling of the central cells, little change could be seen in the female animal which appeared to die of shock.

Probably the earliest histological change was noted in the male animal which died on the 7th day. Every lobule showed marked hydropic degeneration of the periportal cells. They were greatly swollen, with clear cytoplasm and nuclei undergoing karyolysis. The related sinusoids were compressed, but those which were patent contained polymorphs.

More marked changes however were present in the female animal which died on the 6th day. Necrosis was present in every lobule. It affected both central and midzones, but in a few lobules it could be seen that this necrosis originated in two bands which eventually coalesced. The first band occurred paraportally or in the midzone and the necrotic, coagulated cells were in process of disappearing. The cells on either side, immediately central and peripheral to this band were grossly swollen and globular with clear cytoplasm and pyknotic nuclei. Internal to this band was a zone of relatively healthy cells, while immediately around the central vein was another band of necrotic coagulated cells (Fig. 9).

At ten days the necrosis involved both central and midzones as before, while the periportal cells were bloated with clear cytoplasm and pyknotic nuclei, resembling the cells seen in obstetric acute yellow atrophy. Necrosis was very extensive just beneath the liver capsule. Fibrin deposits were present around the necrotic cells. Thrombosis was also seen in some of the portal veins, most of which contained an excess of polymorphs. The hepatic arterioles were grossly dilated, with very thin walls.

In the animal which survived for 13 days there were areas of necrosis in every lobule. They were seen in all parts of the lobule—periportal, midzonal and central. In the last two situations they were generally rounded in shape, whereas in the first site they appeared as rough triangles on section (Fig. 10). The impression received was that the lesions were probably cone-shaped, with their base near the portal tracts. The necrosis was advanced and of coagulative type, but many of the cells were disappearing. A slight polymorphonuclear infiltration was present in some of the necrotic areas. Fibrin deposits occurred in the sinusoids, especially periportally, and thrombosis of the overdistended hepatic arterioles was common.

Kidney.—No noteworthy change could be seen in the kidneys of the female animal which died on the 5th day. Changes were also minimal in the male animal which succumbed on the 7th day. There was marked congestion of one or two glomeruli, with some irregular dilatation of the capillaries. A few eosinophilic casts were present in the distal tubules. Similarly, in the female animal

which died on the 6th day the changes were not gross. Probably the most striking feature was the marked dilatation of the arteries, which were almost of the same calibre as the large veins and almost equally thin-walled. Gross changes were present in the two animals which survived for 10 and 13 days respectively, and they were more marked in the first of these two. The first change to catch the eye was the enormous number of ribbon-shaped eosinophilic casts in the 2nd convoluted tubules and ascending limbs of Henlë (Fig. 11). The epithelium of most of these tubules was necrotic and desquamating, but in non-necrotic areas the epithelium was very flat. Some of the casts contained red blood corpuscles. They were Gram-positive. The proximal convoluted tubules showed gross vacuolation of their lining epithelium, which in some areas had undergone actual necrosis. Faintly Gram-positive globules could be seen in the vacuolated epithelium. The glomeruli presented a variety of appearances. In some there was obvious infarction of a lobule, while in others the capillaries were occluded by hyaline thrombus material (Fig. 12). Some appeared to be merely congested, but on close examination they proved to be completely necrotic. The endothelial cells had disappeared, and only the " ghost " outline of the capillaries was left. The pyknotic remains of polymorph nuclei were seen in these situations. The afferent arterioles were grossly distended and many were thrombosed. Interstitial haemorrhages were common near the infarcted and necrotic glomeruli. The walls of the capillaries in the non-necrotic tufts appeared to be swollen.

DISCUSSION.

It will be seen from the results in Series 1 and 2 that we have confirmed the work of Fauvet (1931), and Végh and von Pallos (1937). Large doses of posterior pituitary extract produced hepatic necrosis and microscopical changes in the kidney. Similarly the work of Byrom (1938) has been partly repeated, and we agree with him that posterior pituitary extract in doses which might reasonably be expected in a natural disease state does not produce lesions resembling those of eclampsia. The presence or absence of the gonads did not seem to affect the outcome.

Our experiments with gonadotrophins, however, fall into another category. Smith and Smith (1939) assumed that the increased serum gonadotrophin which they found in toxaemic patients was of chorionic origin. Browne (1946) showed that synapoidin, a mixture of chorionic and pituitary gonadotrophins, sensitized an individual to the action of vasopressor substances. We have shown that pure chorionic gonadotrophin does not enhance the action of pituitrin, whereas an extract of anterior pituitary does. It has been known for some considerable time that chorionic gonadotrophins differ in some respects from similar substances of pituitary origin, particularly in their action upon the gonads (Ascheim, 1926; Zondek and Ascheim, 1927; Evans and Simpson, 1929; Reïchert, Pencharz, Simpson, Meyer and Evans, 1931, 1932; Engle, 1932–33). Superficially, at least, it would seem that our results indicate a further difference in their power to potentiate the action of pituitrin. We have also demonstrated that urine from some toxaemic patients has a sensitizing action similar to that of anterior pituitary extract. The lesions produced by these methods were always severe, and rather more extensive than those seen in the human disease. Hepatic

necrosis was widespread, but although it was not confined to any particular zone it was most commonly found in periportal situations. The necrosis was accompanied by haemorrhage, and occasionally by fibrinous deposits. Thrombosis was found in hepatic arterioles and portal venules, and many of the arterioles showed a necrotizing lesion similar to that found in the human lesion. Changes in the kidneys resembled fairly closely those found in eclampsia, but as in the liver they tended to be more severe, and in a number of cases verged upon cortical necrosis.

It remains to be decided what possible relationship there may be between the conditions existing in the above experiments and eclampsia. Hofbauer (1918) appears to have been first to suggest that eclampsia was due to overactivity of the pituitary and adrenals. Indirect support was lent to this hypothesis by the observation that hypertension may occur temporarily at adolescence (Janeway, 1913 ; Alvarez, 1920), and that the incidence of hypertension in women increases at the menopause (Riseman and Weiss, 1929), phases of life during which marked hormonal changes occur. Meanwhile, studies in other hypertensive states suggested that they were frequently associated with an excess of substances similar to various anterior pituitary and adrenal hormones (Kylin, 1934, 1935 ; Jones, 1936; Westphal and Sievert, 1937). In 1939, Smith and Smith reported the finding of excessive amounts of gonadotrophins which they assumed were of chorionic origin in the serum of toxaemic patients. There is thus some evidence to indicate that anterior pituitary hormones or similar substances capable of sensitizing the vascular system are present in the blood of toxaemic individuals, and our experiments show that they may also appear in the urine.

The position with regard to the posterior pituitary factor is not so clear. Clark (1929) has shown that pituitrin in anaesthetized animals causes a very temporary rise of blood pressure followed by a distinct fall. Similarly Gruber and Kountz (1930) state that pituitrin has a mixed depressor and pressor action. It has been repeatedly shown that pituitrin is rapidly inactivated *in vivo* and *in vitro* (Heller and Urban, 1935 ; Jones and Schlapp, 1936 ; Page, 1938). According to Dietel (1933) and Schockaert and Lambillon (1937) the blood of normal pregnant women is very effective in this inactivating process. Kustner (1927), however, states that this inhibitory power is not present in the serum of eclamptic patients. In toxaemia there is a retention of chlorides, whereas during the experimental antidiuresis produced by pituitrin the chloride output is increased.

Dieckmann and Michel (1937), who demonstrated an increased sensitivity of toxaemic patients to pituitrin, have summarized the position in the following manner : They suggest that if the syndrome is related to pituitrin, then one of the following conditions must exist : (*a*) an over-secretion, (*b*) a defect in neutralization, (*c*) an increased susceptibility to its action. There is sufficient evidence to indicate that although there may be an over-secretion of posterior pituitary factor in some cases, it is of slight degree. This must be obvious from the fact that the typical effect on chloride excretion is absent. While there may be a defect in neutralization as Kustner suggests, this work will have to be confirmed.

It seems to us that the key to the problem in certain cases of toxaemia lies in the third postulate of Dieckmann and Michel. We would suggest that the vascular system in these cases is sensitized by an excess of secretion from the anterior pituitary, and in that state even normal amounts of pituitrin may be

sufficient to induce hypertension. With conditions such as these the oliguria of toxaemia would be related to the vascular changes, and not to the direct action of the posterior pituitary factor in the renal parenchyma. One would not therefore expect the characteristic change in chloride excretion.

Several points still remain. Although gonadotrophins are the substances most easily recognized by biological tests, it is not certain if they are the hormones responsible for sensitization of the vascular system. They are merely an indication of overaction of the anterior lobe of the hypophysis. It is possible that the gonadotrophins are derived from the placenta in late normal pregnancy, but from the hypophysis in toxaemia. Alternatively, and much more likely, the hormones may be of pituitary origin in both cases, but in toxaemia they are not properly metabolized. Apart from the relationship of these hormones to eclampsia there is one fact of possible practical importance. It would appear from the above experiments that the administration of posterior pituitary extract to patients with a high level of gonadotrophins in the blood is not without danger.

The findings in the kidneys in these experiments raise some interesting possibilities in relation to cortical necrosis and lower nephron nephrosis, but they do not come within the scope of the present communication.

SUMMARY.

1. The administration of large doses of posterior pituitary extract to rabbits results in focal necrosis of the liver, but the lesions do not resemble those of eclampsia.

2. Anterior pituitary extract sensitizes the animal to the action of posterior pituitary extract. Lesions are produced in the liver and kidneys which bear some resemblance to those of eclampsia.

3. Chorionic gonadotrophin does not sensitize the animal to the action of posterior pituitary extract.

4. The urine of many severely toxaemic pregnant women contains a substance which sensitizes the animal to posterior pituitary extract in a manner similar to anterior pituitary extract.

5. It is suggested that toxaemia of late pregnancy may be due to hyperactivity of the anterior pituitary lobe sensitizing the patient to slightly increased amounts of posterior pituitary secretion.

6. It is also suggested that the gonadotrophins in late pregnancy are derived from the anterior lobe of the pituitary.

REFERENCES.

ALVAREZ, W. C.—(1920) *Arch. int. Med. exp.*, **26**, 381.
ANSELMINO, K. J., AND HOFFMAN, F.—(1931*a*) *Arch. f. Gynaek.*, **147**, 597.—(1931*b*) *Ibid.*, **147**, 621.—(1931*c*) *Ibid.*, **147**, 652.
ASCHEIM, S.—(1926) *Z. Geburtsh. Gynäk.*, **90**, 387.
BELL, E. T.—(1932) *Amer. J. Path.*, **8**, 1.
BROWNE, F. J.—(1946) *J. Obstet. Gynaec.*, **53**, 510.
BYROM, F. B.—(1938) *Lancet*, i, 129.
Idem AND WILSON, C.—(1934) *Quart. J. Med.*, **3**, 361.
CLARK, G. A.—(1929) *J. Physiol.*, **68**, 166.
COESTER, C.—(1935) *Z. klin. Med.*, **128**, 665.

COHNHEIM, J.—(1880) ' Vorlesungen über Allgemeine Pathologie.' Berlin.
DE VALERA, E., AND KELLAR, R. J.—(1938) *J. Obstet. Gynaec.*, **45**, 815.
DE WESSELOW, O. L. V. S., AND GRIFFITHS, W. J.—(1934) *Brit. J. exp. Path.*, **15**, 45.
DIECKMANN, W. J., AND MICHEL, H. L.—(1937) *Amer. J. Obstet. Gynaec.*, **33**, 131.
DIETEL, F. G.—(1933) *Klin. Wschr.*, **12**, 1683.
ENGLE, E. T.—(1932–33) *Proc. Soc. exp. Biol., N.Y.*, **30**, 530.
EVANS, A. M., AND SIMPSON, M. E.—(1929) *Amer. J. Physiol.*, **89**, 381.
FAHR, T.—(1924) ' Die Eklampsie.' Bonn.
FAUVET, E.—(1931) *Klin. Wschr.*, **10**, 2125.
GRUBER, C. M., AND KOUNTZ, W. B.—(1930) *J. Pharmacol.*, **39**, 275.
HAM, G. C.—(1941) *J. clin. Invest.*, **20**, 439.
HELLER, H., AND URBAN, F. F.—(1935) *J. Physiol.*, **85**, 502.
HINSELMANN, H.—(1924) ' Die Eklampsie.' Bonn, p. 1.
HOFBAUER, J.—(1918) *Zbl. Gynäk.*, **42**, 745.
HURWITZ, D., AND BULLOCK, L. T.—(1935) *Amer. J. med. Sci.*, **189**, 613.
JANEWAY, T. C.—(1913) *Arch. int. Med.*, **12**, 755.
JONES, A. M., AND SCHLAPP, W.—(1936) *J. Physiol.*, **87**, 144.
JORES, A.—(1936) *Klin. Wschr.*, **15**, 841.
KUSTNER, H.—(1927) *Zbl. Gynäk.*, **51**, 2080.
KYLIN, E.—(1934) *Arch. klin. Med.*, **176**, 301.—(1935) *Ibid.*, **178**, 217.
LEVITT, G.—(1936) *J. clin. Invest.*, **15**, 135.
MUKHERJEE, C.—(1941) *J. Obstet. Gynaec.*, **48**, 586.
MYLIUS, K.—(1928) ' Funktionelle Veranderungen am Gefass system der Netzhaut.' Berlin.
NEVERMANN, H.—(1922) *Zbl. Gynäk.*, **46**, 617.
PAGE, E. W.—(1938) *J. clin. Invest.*, **17**, 207.
REICHERT, F. L., PENCHARZ, R. I., SIMPSON, M. E., MEYER, K., AND EVANS, H. M.—(1931) *Proc. Soc. exp. Biol., N.Y.*, **28**, 843.—(1932) *Amer. J. Physiol.*, **100**, 157.
RISEMAN, J. E. F., AND WEISS, S.—(1929) *Amer. Heart J.*, **5**, 172.
SCHOCKAERT, J. A., AND LAMBILLON, J.—(1937) *C.R. Soc. Biol., Paris*, **122**, 478.
SMITH, G. V. S., AND SMITH, O. W.—(1939) *Amer. J. Obstet. Gynaec.*, **38**, 618.—(1940) *Ibid.*, **39**, 405.
TEEL, H. M., AND REID, D. E.—(1939) *Endocrinology*, **24**, 297.
THEOBALD, G. W.—(1934) *Clin. Sci.*, **1**, 225.
VÉGH, L., AND VON PALLOS, K.—(1937) *Klin. Wschr.*, **16**, 1536.
VOLHARD, F.—(1918) ' Die doppelseitingen Haematogenen Nierenerkrankungen.' Berlin.
WAGENER, H. P.—(1933) *J. Amer. med. Ass.*, **101**, 1380.
WESTPHAL, K., AND SIEVERT, C.—(1937) *Z. klin. Med.*, **133**, 223.—(1937) *Ibid.*, **133**, 342.
ZONDEK, B., AND ASCHEIM, S.—(1927) *Arch. Gynäk.*, **130**, 1.

THE USE OF THE INDIRECT SENSITIZATION TECHNIQUE AS A METHOD OF TITRATING ANTIBODIES AGAINST A CHEMICAL COMPOUND.

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SPECIFIC serum antibodies against determinant groups such as atoxyl may be produced by inoculating rabbits with the compound coupled by the azo linkage with a foreign protein (Landsteiner, 1945). Antibodies produced in this way against atoxyl are usually demonstrated by means of the precipitation or complement-fixation reactions. For the *in vitro* test the atoxyl is diazotized and coupled to a protein immunologically distinct from that used for the inoculations. Further, the precipitation reaction may be specifically inhibited by a solution of the chemical compound itself.

The purpose of the present paper is to record experiments on the use of the agglutination technique as a method of titrating serum antibodies against atoxyl. This method in our hands has proved not only more sensitive than the precipitation technique for this purpose, but has also proved more easy to standardize.

The increased sensitivity of the agglutination reaction over that of the precipitation reaction has long been recognized, and the method of adsorbing the precipitinogen on to an agglutinable surface has been employed by many workers, and forms the rationale of the collodion particle technique described by Cannon and Marshall (1940).

Pressman, Campbell and Pauling (1942) demonstrated the agglutinative property of antisera against atoxyl by using as the agglutinogen atoxyl coupled directly to rabbit red cells by means of the diazo reaction. However, cells treated in this way were stable for only a relatively short time after this treatment, although they were stable for a sufficient period for the performance of the test.

After the development of the antiglobulin sensitization test (Coombs, Mourant and Race, 1945) for the detection of " incomplete " non-agglutinating antibodies adsorbed on a cell surface, the idea presented itself of using a modified version of this technique as a means of titrating antibodies against determinant groups such as atoxyl.

Eagle, Smith and Vickers (1936) showed that atoxyl and other chemical compounds could be coupled to serum proteins to a moderate degree without interfering with the specific combining property of the antibodies in the serum. For the present purpose atoxyl was coupled to the proteins of human sera containing the D Rh " incomplete " antibody. The coupling of up to 15 groups of atoxyl per protein molecule did not interfere significantly with the specific combining property of the antibody for the homologous red cells. Cells, after being incubated with such a treated serum, adsorbed the coupled Rh " incomplete "

antibodies and, after thorough washing in saline, formed a stable red cell suspension which could be agglutinated specifically by antisera prepared against atoxyl as well as by antisera prepared against human serum globulin.

For use with this system of Rh-positive human cells sensitized with the D Rh " incomplete " antibody coupled with atoxyl, the rabbit serum prepared against atoxyl required absorption in order to remove antibodies reacting with normal human cells and with human globulin if antibodies reactive with human globulin were present in the serum.

When it was subsequently found (Gleeson-White, Heard, Mynors and Coombs 1950) that some rabbit anti-bovine red cell sera would sensitize bovine red cells without agglutinating them and that this sensitization of the red cells of certain oxen could be detected by the antiglobulin sensitization technique, another system for the present purpose became available. It will be shown that bovine red cells sensitized with rabbit anti-bovine red cell antibodies coupled with atoxyl formed a stable suspension which, after being thoroughly washed in saline, could be agglutinated by rabbit antisera produced against atoxyl. In this system no previous absorptions of the rabbit anti-atoxyl sera were necessary, because rabbit sera contain no naturally occurring agglutinins against bovine red cells, nor do the rabbit anti-atoxyl sera contain antibodies reactive against the rabbit globulin sensitizing the bovine red cells.

Using these two systems, rabbit antisera have been tested for their content of antibodies reacting with atoxyl.

MATERIALS AND METHODS.

Preparation of p-phenylarsonic diazonium chloride.

The required amount of atoxyl was dissolved in three equivalents of hydrochloric acid solution and cooled to 0° C. One equivalent of cooled sodium nitrite solution was added, and after a few minutes standing in the cold the solution was partially neutralized by the addition of solid sodium bicarbonate until a faint yellow colour appeared. This colour is not due to self-coupling of the diazonium compound, and disappears completely if the solution is again made acid. The neutralized diazonium salt was added rapidly with stirring to the buffered protein solution.

Atoxyl-azo-egg-albumin.

Four per cent thrice crystallized egg-albumin dissolved in M/10 phosphate buffer pH 7·6 received 15 molar equivalents of diazonium salt. It was left for 12 hours at 4° C., and then dialyzed first against buffer and then against saline for three days. Very little colour was lost to the outside liquid on the first day and none on the following days.

Atoxyl-azo-serum proteins.

In the coupling of p. phenylarsonic diazonium chlroide to the proteins of serum, we assumed an average molecular weight of 100,000 for the proteins and a concentration of 5 per cent. Thus we assumed the serum to be a 5×10^{-4} molar protein solution. The number of molecules of azo-compound per molecule protein given in this paper was calculated on this basis.

Coupling of the p. phenylarsonic diazonium chloride to a concentrated protein solution at a pH higher than 7 is almost quantitative provided the diazonium salt is not in excess and the values for the ratio, molecule azo-compound per molecule protein, given are probably correct to within 10 to 20 per cent.

It has been assumed that the diazonium salt couples equally well with all protein molecules of the serum, and that the " conjugated " antibody molecules have the same number of azo-groups as the other serum proteins. No good evidence for such a homogeneous distribution can be given, but it seems plausible in view of the similar reactivity offered by the different proteins, not only to diazonium salts but to many other reagents (Herriott, 1947).

Coupling of the proteins of human sera containing the D Rh " incomplete " antibody and rabbit anti-bovine red cell sera for the in vitro *tests.*

The neutralized p. phenylarsonic diazonium chloride solution was added to the sera without any further addition of buffer. After 24 hours at 4° C. the sera were dialysed against saline for 36 hours. The sera were then preserved at −20° C. until required for the serological tests.

Production of rabbit antisera against atoxyl-azo-egg-albumin.

Three rabbits (No. 1733, 1693 and 1556) received seven inoculations at three-day intervals of 2·5 ml. each of the atoxyl-azo-egg-albumin antigen described above. The inoculations were given intraperitoneally, and the animals were bled for serum on the 9th day after the last inoculation.

For brevity these sera have been described as anti-atoxyl sera throughout the paper.

Rabbit anti-bovine red cell sera.

Six rabbits each received five intravenous injections of 1·0 ml. of a 10 per cent suspension of washed bovine red cells at three-day intervals, and were bled for serum on the 9th day following the last inoculation.

Rabbit anti-human-globulin serum.

This was prepared as described by Coombs and Mourant (1947).

TABLE I.—*The Coupling of p. Phenylarsonic Diazonium Chloride to the Proteins of a Human Combining Power of the Antibody*

A 1 per cent suspension of Rh-positive human red cells was added in
After incubation the cells were washed in

Anti-D Rh sera coupled with diazotized atoxyl being titrated.	To the first set was added an equal volume of normal rabbit serum 1/10.							
Serum dilutions:—	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	Saline.
Serum untreated	—	—	—	—	—	—	—	—
„ coupled 2 groups/mol.	—	—	—	—	—	—	—	—
„ „ 5 „	—	—	—	—	—	—	—	—
„ „ 10 „	—	—	—	—	—	—	—	—
„ „ 15 „	—	—	—	—	—	—	—	—
„ „ 20 „	—	—	—	—	—	—	—	—
„ „ 25 „	—	—	—	—	—	—	—	—

Key to this and subsequent tables: ++ Strong agglutination.

Goat anti-rabbit-globulin serum.

This serum was produced by inoculating a goat with normal rabbit serum as described by Heard, Hinde and Mynors (1949). Before being used in the indirect sensitization tests to detect rabbit antibodies adsorbed on bovine red cells, the inactivated serum was absorbed free of agglutinins reactive against bovine cells.

EXPERIMENTAL STUDY.

Experiment I.—The coupling of p. phenylarsonic diazonium chloride to the proteins of a human serum containing the D Rh "incomplete" antibody, and the effect of this conjugation on the combining power of the antibody with the homologous antigen.

Six samples of a human serum containing the Rh antibody were coupled with diazotized atoxyl as described under *Methods* on a calculated basis of 2, 5, 10, 15, 20 and 25 groups of atoxyl per protein molecule respectively. A further sample of the serum was left untreated to serve as a control. All the sera were dialysed against saline for 48 hours to remove any uncombined diazonium salt.

The seven serum samples were then titrated against an equal volume of a 1 per cent suspension of Group O Rh positive human red cells. After the mixture had been incubated for half-an-hour at 37° C. the cells were washed three times in saline, and then tested for antibody sensitization by a suitable dilution of a rabbit anti-human-globulin serum and a rabbit anti-atoxyl serum ; a dilution of normal rabbit serum served as a control. These three sera had been absorbed previously with well-washed human red cells ; the anti-atoxyl serum had also been neutralized with human globulin. The mixtures were incubated for a further one hour at 37° C., and the sedimented cells read for agglutination on a glass slide under the low-power microscope. The results are shown in Table I.

Although by this technique it is not possible to measure directly any reduction in the sensitizing power of the anti-D serum due to the coupling, it is apparent that antibody coupled with as many as 25 groups of atoxyl still had considerable combining power, the sensitization being detected by the anti-globulin and anti-atoxyl sera to a titre of 32.

The rabbit anti-atoxyl serum had no demonstrable action either on cells sensitized with untreated Rh "incomplete" antibodies or on cells previously

Serum containing the D Rh "Incomplete" Antibody, and the Effect of this Conjugation on the with the Homologous Antigen.

equal volumes to titrations of the coupled "incomplete" anti-Rh sera.
saline and divided into three parallel sets.

To the second set was added an equal volume of rabbit anti-human-globulin serum 1/20.								To the third set was added an equal volume of rabbit anti-atoxyl serum 1/10.							
1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	Saline.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	Saline.
++	++	++	++	++	++	−	−	−	−	−	−	−	−	−	−
++	++	++	++	++	++	−	−	−	−	−	−	−	−	−	−
++	++	++	++	++	−	−	−	++	++	++	−	−	−	−	−
++	++	++	++	++	−	−	−	++	++	++	++	−	−	−	−
++	++	++	++	++	−	−	−	++	++	++	++	−	−	−	−
++	++	++	++	−	−	−	−	++	++	++	++	−	−	−	
++	++	++	++	−	−	−	−	++	++	++	++	−	−	−	

\+ Weak agglutination. — No agglutination.

exposed to normal human serum conjugated with diazotized atoxyl. Cells sensitized with the coupled Rh " incomplete " antibodies remained stable in the presence both of saline and of a 1 in 10 dilution of normal rabbit serum.

Experiment II.—A solution of atoxyl will neutralize the agglutinating action of a rabbit anti-atoxyl serum but not a rabbit anti-human-globulin serum on human red cells sensitized with Rh " incomplete " antibody conjugated with diazotized atoxyl.

The experiment was performed as summarized in Table II. Four similar titrations of a rabbit anti-human-globulin serum were set up in 0·1 ml. amounts with an initial dilution of 1 in 8. An equal volume of saline was added to each tube of Racks 1 and 2, while to each tube of Rack 3 was added 0·1 ml. of a 0·5 per cent solution of atoxyl in saline, and to each tube of Rack 4, 0·1 ml. of a normal human serum diluted 1 in 4.

After incubating the mixtures at 37° C. for half-an-hour, 0·1 ml. of a 1 per cent suspension of Rh positive Group O human red cells was added to each tube of Rack 1, while to each tube of Racks 2, 3 and 4 was added 0·1 ml. of a 1 per cent suspension of the human cells sensitized with the Rh " incomplete " antibody conjugated with atoxyl.

This sensitized red cell suspension had been prepared previously by incubating a volume of a 1 per cent suspension of human red cells with an equal volume of a 1 in 5 dilution of the human " incomplete " anti-Rh serum which had been coupled with diazotized atoxyl on a calculated basis of 15 groups per protein molecule. After half-an-hour at 37° C. for sensitization, the cells were centrifuged down and, after discarding the supernatant fluid, were washed twice in saline and finally resuspended to form a 1 per cent suspension.

Another set of 4 racks was set up in a similar manner except that in this case the serum titrated was a rabbit anti-atoxyl serum starting at a dilution of 1 in 2, instead of a rabbit anti-human-globulin serum.

After a final incubation of all the tubes at 37° C. for one and a half hours, the sedimented red cells were read for agglutination in the usual manner.

From the results shown in Table II it may be seen that the agglutination of the human cells sensitized with " incomplete " Rh antibody coupled with diazotized atoxyl, by the rabbit anti-human-globulin serum was inhibited by normal human serum but not by a solution of atoxyl, while the agglutination of these cells by the rabbit anti-atoxyl serum was inhibited by the solution of atoxyl but not by normal human serum. This shows the two distinct actions of the two sera in bringing about the agglutination of the sensitized cell complex.

Experiment III.—The coupling of p. phenylarsonic diazonium chloride to the proteins of a rabbit anti-bovine red cell serum, and the effect of this conjugation on the combining power of the antibody with bovine red cells.

Twenty ml. of a rabbit anti-bovine red cell serum, heat inactivated at 56° C. for half-an-hour, were divided into 5 samples of 4 ml. each. Diazotized atoxyl was coupled with the proteins of one sample on a calculated basis of 5 groups per protein molecule. Three further samples were likewise treated on a basis of 15, 25 and 40 groups atoyxl per protein molecule respectively. The fifth sample was untreated and served as a control.

TABLE II.—*A Solution of Atoxyl will Neutralize the Agglutinating Action of a Rabbit Anti-atoxyl Serum, but not a Rabbit Anti-human-globulin Serum on Human Red Cells Sensitized with Rh "Incomplete" Antibody Conjugated with Diazotized Atoxyl.*

Rack.	Incubated for 30 minutes at 37° C. for neutralization.		Subsequently added.	Dilutions of rabbit anti-human-globulin serum.								
	Serum being titrated.	Neutralization.	1% cell suspension.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	1:1024.	Saline.
1	Rabbit anti-human-globulin serum	Saline	Norman human cells	−	−	−	−	−	−	−	−	−
2	Do.	,,	Human cells sensitized with "incomplete" Rh antibody conjugated with diazotized atoxyl	++	++	++	++	++	++	+	−	−
3	,,	0·5% solution of atoxyl		++	++	++	++	++	++	+	−	−
4	,,	Human serum 1/4		−	−	−	−	−	−	−	−	−

				Dilutions of rabbit anti-atoxyl serum.								
				1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	Saline.
1	Rabbit anti-atoxyl serum	Saline	Normal human cells	−	−	−	−	−	−	−	−	−
2	Do.	,,	Human cells sensitized with "incomplete" Rh antibody conjugated with diazotized atoxyl	++	++	++	++	++	++	+	−	−
3	,,	0·5% solution of atoxyl		−	−	−	−	−	−	−	−	−
4	,,	Human serum 1/4		++	++	++	++	++	++	+	−	−

The five samples were then tested in a similar manner to those in Experiment I by the indirect sensitization technique for their ability to sensitize the red cells of ox 5153. The cells of this animal belonged to the "markedly agglutinable" class of bovine red cells (Gleeson-White *et al.*, 1950). The sensitization of the bovine red cells was tested for by the use of a suitably absorbed goat anti-rabbit-globulin serum as well as by a rabbit anti-atoxyl serum; the pre-inoculation serum of this rabbit served as a control. The experiment is summarized in Table III, which also shows the results of the tests.

The slight degree of agglutination of the sensitized cells resuspended in normal rabbit serum which occurred with untreated anti-bovine red cell serum, and with that serum after coupling with five groups of atoxyl, corresponded with the direct agglutinating activity of this serum for these cells. This activity was removed when the serum was coupled with 15 groups of diazotized atoxyl per protein molecule.

It is clear from the results in Table III that the rabbit anti-atoxyl serum had no agglutinating action on bovine cells sensitized with the untreated anti-bovine red cell antiserum. The results also show that increasing the number of atoxyl groups per antibody molecule apparently reduced the sensitizing capacity of the antibody. However, the coupling of 5 and 15 groups per molecule did not interfere significantly with the combining power of the antibody with the homologous cells. Cells sensitized with antibody coupled with 15 groups of p. phenylarsonic diazonium chloride were stable in saline or the dilution of normal rabbit serum, but were agglutinated by the rabbit anti-atoxyl serum as well as by the goat anti-rabbit-globulin serum.

Experiment IV.—A solution of atoxyl will neutralize the agglutinating action of a rabbit anti-atoxyl serum, but not a goat anti-rabbit-globulin serum on bovine red cells sensitized with a rabbit anti-bovine red cell antibody conjugated with diazotized atoxyl.

The procedure of this experiment was the same as that described in Experiment II with the differences indicated in Table IV.

The sensitized cells for this experiment were prepared by incubating a volume of a 1 per cent suspension of bovine red cells with an equal volume of a 1 in 20 dilution of a rabbit anti-bovine red cell serum which had been coupled with p. phenylarsonic diazonium chloride on a calculated basis of 15 groups per protein

TABLE III.—*The Coupling of p. Phenylarsonic Diazonium Chloride to th Prote Conjugation on the Combining Pow of the*

A 1 per cent suspension of bovine red cells was added in equ volun coupled with varying amounts of atoxyl. After an incubatio perio saline and divided int three

Rabbit anti-bovine red cell sera coupled with diazotized atoxyl being titrated.	To the first set was added an equal volume of normal rabbit serum diluted 1 in 10.								
Serum dilutions:—	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	Salin	1:4.
Serum untreated	+	+	−	−	−	−	−	−	−	++
„ coupled 5 groups/mol.	+	+	−	−	−	−	−	−	−	++
„ „ 15 „	−	−	−	−	−	−	−	−	−	++
„ „ 25 „	−	−	−	−	−	−	−	−	−	++
„ „ 40 „	−	−	−	−	−	−	−	−	−	++

molecule. After half-an-hour at 37° C. for sensitization the cells were centrifuged down and, after discarding the supernatant fluid, were washed twice in saline and finally resuspended in saline to give a stable 1 per cent suspension.

The results in Table IV show that the agglutination of bovine red cells sensitized with the rabbit antibody coupled with p. phenylarsonic diazonium chloride by the goat anti-rabbit-globulin serum was inhibited by normal rabbit serum, but not by the solution of atoxyl, while the agglutination of these cells by the rabbit anti-atoxyl serum was inhibited by the solution of atoxyl but not by normal rabbit serum. This again shows the two distinct actions of the two sera in bringing about the agglutination of the sensitized cell complex.

Experiment V.—Titration of the pre- and post-inoculation sera of three rabbits inoculated with atoxyl-azo-egg-albumin for their antibody content against atoxyl.

The three rabbit sera were titrated against an equal volume of a 1 per cent suspension of washed bovine red cells sensitized with rabbit antibody coupled with 15 groups of p. phenylarsonic diazonium chloride. The preparation of this sensitized red cell suspension was the same as that described in Experiment IV.

From Table V it may be seen that none of the pre-inoculation sera showed any activity while the titres of the anti-atoxyl activity in the post-inoculation sera were 128, 128 and 64 respectively.

None of the sera agglutinated normal bovine cells or cells sensitized with untreated rabbit antibody or rabbit antibody coupled with 15 groups of diazotized 1-amino-naphthalene-4 sulphonic acid. In addition, all the reactions were inhibited by a previous neutralization of the antisera with an equal volume of a 0·5 per cent solution of atoxyl.

The three sera were also examined by the interfacial precipitation technique. The sera were used undiluted, and the testing antigen consisted of horse serum proteins coupled with 15 groups of p. phenylarsonic diazonium chloride per protein molecule. The antigen was used at dilutions of 1 in 10, 1 in 100, and 1 in 1000. Normal horse serum in similar dilutions served as a control for non-specific reactions with the protein carrier. The tests were observed over a period of five and a half hours at room temperature. Serum 1693 gave a weak but unequivocal reaction ; the other two sera gave negative results.

oride to th*Proteins of a Rabbit Anti-bovine Red Cell Serum, and the Effect of this*
ing Pow*of the Antibody with Bovine Red Cells.*

led in equ volumes to titrations of the rabbit anti-bovine red cell sera
incubatio period for sensitization the cells were washed three times in
divided int three parallel sets.

of normal		To the second set was added an equal volume of a goat anti-rabbit-globulin serum diluted 1 in 5.										To the third set was added an equal volume of a rabbit anti-atoxyl serum diluted 1 in 10.								
1:512.	Saline.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	Saline.		1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	Saline.
−	−	++	++	++	++	++	++	++	−	−	.	+	+	−	−	−	−	−	−	−
−	−	++	++	++	++	++	++	−	−	−	.	++	++	++	++	++	++	−	−	−
−	−	++	++	++	++	++	+	−	−	−	.	++	++	++	++	++	++	−	−	−
−	−	++	++	++	++	−	−	−	−	−	.	++	++	++	++	+	−	−	−	−
−	−	++	++	+	−	−	−	−	−	−	.	++	++	+	−	−	−	−	−	−

Experiment VI.—The smallest concentration of atoxyl which will inhibit 4 agglutinating doses of a rabbit anti-atoxyl serum.

Two parallel rows of " doubling dilutions " of atoxyl were set up in 0·1 ml. amounts, beginning each titration with a 0·5 per cent solution of atoxyl in saline. To each tube of the first row was added 4 agglutinating doses of a rabbit anti-atoxyl serum (0·1 ml. serum diluted 1 in 32), and to each tube of the second row was added 4 agglutinating doses of a goat anti-rabbit-globulin serum (0·1 ml. serum diluted 1 in 8). The contents of the tubes were mixed and allowed to stand for half-an-hour at 37° C.

To each tube of both rows was then added 0·1 ml. of a 1 per cent suspension of washed bovine cells sensitized with rabbit antibody conjugated with 15 groups of diazotized atoxyl per molecule prepared as described in Experiment IV. After a further incubation for one and a half hours at 37° C. the cell deposits were examined for agglutination.

Two further racks were set up in the same way except that the material titrated was the sodium salt of 1-amino-naphthalene-4 sulphonic acid (sodium naphthionate). The experiment and the results are summarized in Table VI.

It may be seen that 4 agglutinating doses of the anti-serum against atoxyl were inhibited by a concentration of 0·015 per cent (1 in 32 of 0·5 per cent) of atoxyl, a 7×10^{-4} molar solution. The concentrations tested brought about no inhibition of agglutination by the goat anti-rabbit-globulin serum. The rabbit anti-atoxyl serum was not inhibited by the concentrations of sodium naphthionate tested.

GENERAL OBSERVATIONS.

The following criteria are those which we believe must be satisfied if the reactions observed with this technique are to be considered specific for the chemical compound under investigation.

First, normal sera should exert no agglutinating action against the testing cell suspension. Second, the antisera produced in response to the inoculations and after any necessary absorptions should not react with unsensitized cells, or cells sensitized with untreated antibody. Finally, the reaction between the anti-serum and cells sensitized with the appropriately conjugated antibody should be inhibited specifically by a solution of the chemical compound under consideration. The experiments reported here satisfy these criteria.

In other experiments a series of rabbits were inoculated with alum-precipitated horse serum conjugated with diazotized 1-amino-naphthalene-4 sulphonic acid. The post-inoculation sera of these rabbits, at a low titre, agglutinated bovine red cells sensitized with rabbit antibody conjugated with this chemical compound, but the reaction was not inhibited by a solution of the chemical compound. Furthermore, the sera, although giving no reaction with unsensitized cells, brought about very weak agglutination with bovine cells sensitized with untreated antibody. The mechanism involved in these reactions has not, as yet, been ascertained, but it was clear that the reactions could not be regarded as specific for the compound concerned.

Three further rabbits receiving a course of inoculations of egg albumin conjugated with 15 groups of diazotized 1-amino-naphthalene-3:8 disulphonic acid failed to produce antibodies against this chemical compound detectable either by the technique described or by the precipitation reaction.

TABLE IV.—*A Solution of Atoxyl will Neutralize the Agglutinating Action of a Rabbit Anti-atoxyl Serum, but not a Goat*

TABLE IV.—*A Solution of Atoxyl will Neutralize the Agglutinating Action of a Rabbit Anti-atoxyl Serum, but not a Goat Anti-Rabbit-Globulin Serum on Bovine Red Cells Sensitized with a Rabbit Anti-bovine Red Cell Antibody Conjugated with Diazotized Atoxyl.*

Rack.	Incubated for 30 minutes at 37° C. for neutralization.		Subsequently added.	Dilutions of goat anti-rabbit-globulin serum absorbed with normal bovine cells.									
	Serum being titrated.	Neutralization.	1% cell suspension.	1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	Saline.
1	Goat anti-rabbit-globulin serum absorbed with normal ox cells	Saline	Normal bovine cells	−	−	−	−	−	−	−	−	−	−
2		,,	Bovine cells sensitized with rabbit antibody conjugated with diazotized atoxyl	++	++	++	++	++	+	−	−	−	−
3		0·5% solution of atoxyl		++	++	++	++	++	+	−	−	−	−
4		Normal rabbit serum 1/10		−	−	−	−	−	−	−	−	−	−

				Dilutions of rabbit anti-atoxyl serum.									
				1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	Saline.
1	Rabbit anti-atoxyl serum	Saline	Normal bovine cells	−	−	−	−	−	−	−	−	−	−
2	,,	,,	Bovine cells sensitized with rabbit antibody conjugated with diazotized atoxyl	++	++	++	++	++	++	++	−	−	−
3	,,	0·5% solution of atoxyl		−	−	−	−	−	−	−	−	−	−
4	,,	Normal rabbit serum 1/10		++	++	++	++	++	++	++	−	−	−

TABLE V.—*Titration of the Pre- and Post-Inoculation Sera of Three Rabbits Inoculated with Atoxyl-Azo-Egg-Albumin for their Antibody Content against Atoxyl.*

Sera being titrated.		Cell suspension.	Serum dilutions.									
			1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	1:1024	Saline.
Rabbit 1733	Pre-inoculation	1 per cent bovine red cell suspension sensitized with rabbit antibody coupled with 15 groups of diazotized atoxyl, and subsequently washed free of all serum	−	−	−	−	−	−	−	−	−	−
	Post- ,,		++	++	++	++	++	++	−	−	−	−
,, 1693	Pre- ,,		−	−	−	−	−	−	−	−	−	−
	Post- ,,		++	++	++	++	++	++	−	−	−	−
,, 1556	Pre- ,,		−	−	−	−	−	−	−	−	−	−
	Post- ,,		++	++	++	++	++	−	−	−	−	−

TABLE VI.—*The Smallest Concentration of Atoxyl which will Inhibit 4 Agglutinating Doses of a Rabbit Anti-atoxyl Serum.*

Incubated for 30 minutes at 37° C.		Subsequently added.	Titrations of atoxyl and sodium naphthionate; initial concentration 0·5 per cent.									
Chemical being titrated.	Antisera; 4 agglutinating doses.			1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	Saline.
Atoxyl (sodium salt)	Rabbit anti-atoxyl	Bovine cells sensitized with rabbit antibody conjugated with diazotized atoxyl		−	−	−	−	−	+	++	++	++
,, ,,	Goat anti-rabbit-globulin			++	++	++	++	++	++	++	++	++
Sodium naphthionate	Rabbit anti-atoxyl			++	++	++	++	++	++	++	++	++
,, ,,	Goat anti-rabbit globulin			++	++	++	++	++	++	++	++	++

The atoxyl hapten was the only one of those investigated in this study against which demonstrable antibodies were successfully produced. Nevertheless, the method described here for the detection of such antibodies should prove of general application.

SUMMARY AND CONCLUSIONS.

A technique has been evolved which permits antisera produced against atoxyl-azo-egg-albumin to be titrated for their anti-atoxyl activity by means of an agglutination reaction.

The method depends on obtaining a stable agglutinable suspension of red cells sensitized with antibodies conjugated with diazotized atoxyl. Such a suspension may then be agglutinated by antisera reactive against atoxyl.

Either human cells sensitized with conjugated " incomplete " Rh antibody or the cells of certain oxen sensitized with conjugated rabbit antibodies may be employed to furnish the stable sensitized cell suspension.

Using this technique the anti-atoxyl activities of three rabbit antisera have been estimated ; in each case the reaction was specifically inhibited by a solution of atoxyl.

The method described for the demonstration of antibodies reactive against atoxyl should prove suitable for the investigation of antibodies against other chemical compounds.

We would like to express our thanks to Professor A. Wormall for supplying us with the crystalline egg albumin used in these experiments, and for his encouragement in this work.

REFERENCES.

CANNON, P. R., AND MARSHALL, C. E.—(1940) *J. Immunol.*, **38**, 365.

COOMBS, R. R. A., AND MOURANT, A. E.—(1947) *J. Path. Bact.*, **59**, 105.

Iidem AND RACE, R. R.—(1945) *Brit. J. exp. Path.*, **26**, 255.

EAGLE, H., SMITH, D. E., AND VICKERS, P.—(1936) *J. exp. Med.*, **63**, 617.

GLEESON-WHITE, M. H., HEARD, D. H., MYNORS, L. S., AND COOMBS, R. R. A.—(1950) *Brit. J. exp. Path.*, **31**, 321.

HEARD, D. H., HINDE, I. T., AND MYNORS, L. S.—(1949) *J. Hyg., Camb.*, **47**, 119.

HERRIOTT, R. M.—(1947) 'Advances in Protein Chemistry,' vol. iii. New York (Academic Press), p. 169.

LANDSTEINER, K.—(1945) 'The Specificity of Serological Reactions,' revised ed., Cambridge, Mass. (Harvard University Press).

TOCOPHEROL AND PROTEIN DEFICIENCY IN RELATION TO THE DEVELOPMENT OF DIETETIC MASSIVE NECROSIS OF THE LIVER IN RATS.*

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IT is now established that supplements of either cystine alone or of tocopherol alone will completely protect against the development of massive hepatic necrosis in rats receiving a diet deficient both in tocopherol and sulphur-containing amino-acids (thio-amino-acids) (György, 1947; Glynn, Himsworth and Neuberger, 1945). The discovery that a double dietary deficiency was required to produce this lesion did much to remove the discrepancies between the results in different laboratories. Nevertheless there are still considerable differences between workers in the certainty and speed with which they produce this lesion and, moreover, a few still report that, although they use the same diet which readily produces hepatic necrosis elsewhere, they are unable to produce liver lesions in their animals. Knowing the tenacity with which the body holds to its stores of tocopherol, it occurred to us that the composition of the pre-experimented diet might influence the results when, subsequently, the necrogenic diet was given. A preliminary experiment indicated that there was substance in this suggestion (Himsworth and Lindan, 1949). The present investigation was, therefore, undertaken to establish the facts with regard to tocopherol deficiency, and to see whether the poverty or richness of the pre-experimental diet in respect of this and other dietary components was also of importance.

METHODS.

Animals.

About 500 albino (Wistar strain) male and female rats were used. They were kept 2 to 4 in a cage.

Diets and supplements.

Three diets were used, two deficient in tocopherol only, the third deficient in both tocopherol and, as judged by biological tests, in thio-amino-acids. The two diets deficient only in tocopherol differed in their protein composition; one containing a 15 per cent casein and 4 per cent yeast protein, the other 25 per cent casein. As the relevant feature of these two diets was their low tocopherol content, they will be referred to as tocopherol-deficient diets. In the third diet deficient both in tocopherol and in thio-amino-acids dried baker's yeast (from The Distillers Co., Ltd., Edinburgh) was the only source of protein. A word

* Work undertaken for the Medical Research Council.

must be said about the justification for continuing to regard this latter diet as deficient in thio-amino-acids.

When this 18 per cent yeast diet was first used, the available analyses indicated that the yeast was poor in thio-amino-acids. Recent analyses by improved methods (Lindan and Work, 1950) have shown that it is considerably richer than was thought. Yet rats taking a diet containing this yeast as the only source of protein still developed acute hepatic necrosis, just like animals taking a synthetic mixture of amino-acids which did not include any sulphur-containing amino-acids (Glynn, Himsworth and Neuberger, 1945), and supplements of cystine or methionine still prevent this lesion developing. In other words, although, chemically speaking, the yeast contained sufficient thio-amino-acids, biologically speaking it appeared deficient in these. It is in that sense that the term low tocopherol thio-amino-acid deficient is applied to the yeast diet. The recent analyses have also revealed that the yeast diet contains 7 per cent of yeast protein, and not 8 per cent as had previously been believed.

The detailed composition of all diets is shown in Table I. The olive oil and cod liver oil were mixed saily into the diets, as were the following water-soluble vitamins.

TABLE I.—*Composition of Diets.*

	Low tocopherol diet.		Low tocopherol and low thio-amino-acid diet (necrogenic yeast diet) (per cent.)
	(*a*) Moderate casein (per cent.)	(*b*) High casein (per cent.)	
Casein	15	25	—
Yeast, dried	10	—	18
Corn starch	67	67	73
Salt mixture (Glaxo)	3	3	3
Olive oil (B.P.)	5	5	5
Cod-liver oil	1	1	1

To all diets the following supplements of water-soluble vitamins were added daily: Thiamin HCl 20 μg., pyridoxin 20 μg., riboflavin 25 μg., calcium pantothenate 100 μg.

Tocopherol content of diets.

Tocopherol was estimated chemically (method of Tosic and Moore, 1945) not only in olive oil but also in casein and corn starch, because an extraction with hot ethanol for 2 hours under reflux yielded 1·5 g. of fat per 100 g. casein and 0·5 g. maize oil per 100 g. corn starch. Table I*a* gives the values for total ferric chloride-

TABLE I*a*.—*Tocopherol Content in Dietary Fats.*

	Total $FeCl_3$ reducing substances (mg./100 g.).	α-Tocopherol (mg./100 g.).
Olive oil	30	20
Maize oil	50	25
Casein fat	11	Not estimated

reducing substances and a fraction corresponding to α-tocopherol in the olive oil, maize oil and casein fat. The tocopherol content was roughly the same in all three diets, and amounted to 1·1 mg. per 100 g. of diet, the olive oil providing

1 mg. It is believed that there was no tocopherol in the yeast (György, 1948, private communication).

The presence of small amounts of tocopherol in the two casein diets (15 per cent and 25 per cent casein respectively) was also confirmed biologically. Female rats reared on the casein diets from the 12th day of life grew normally, went through gestation without any resorption and littered, but many of their young showed at the end of the lactation period the typical symptoms of tocopherol deficiency, such as convulsions and paralysis of hind legs. It seems therefore that this trace of tocopherol in the casein diets was sufficient for normal growth and gestation in the mother, but inadequate to ensure normal supplies to the litter. Male rats were also reared on these casein diets and then mated with normal females fed on stock diet. Normal pregnancies resulted, proving that the casein diets contained enough tocopherol for normal testicular development of the male rat.

The casein diets were always given *ad libitum*. The yeast diet was given *ad libitum* to weanlings only, and was restricted to 8 g. per rat per day for older animals when they were able to consume more than that amount.

Supplements.

In order to examine the protective action of vitamin E against liver necrosis α-tocopherol acetate (33 per cent solution in arachis oil, Roche Products Ltd.) was given to some groups of rats fed on casein or yeast diets. Supplements of tocopherol were given to rats individually by mouth except in one experiment when tocopherol was mixed with the casein diet.

In the experiments in which cystine was given this was added to the yeast diet in amounts of 0·63 g. of L-cystine per 100 g. of diet—that is, 50 mg. in an 8-g. portion.

Criteria for significance.

The efficacy of any particular régime in promoting the development of acute hepatic necrosis is shown by the speed with which this lesion develops. This can be expressed by the mean time of death after the particular régime is started in a given group of animals. Differences between means greater than three times the standard error of the difference have been taken as significant. This procedure is quite satisfactory for experiments in which all the animals die or all survive. When, however, only a proportion die, a better appreciation of the significance can be obtained from a graph of the percentage cumulative mortality against time.

RESULTS.

(a) *Pilot experiment. Demonstration of the effect of the composition of the diet in the "pre-experimental" (preparatory) period on the subsequent development of dietetic massive necrosis, the complete protection offered by supplements of cystine alone or tocopherol alone, and the degree of protection which continued after withdrawal of these supplements.*

The results of this experiment are summarized in Table II.

TABLE II.—*Protective Effect of Tocopherol or Cystine Supplementation against Liver Necrosis.*

Group.	Number of rats.	Low tocopherol casein diet. 1–107th day. Supplements.	Low tocopherol yeast diet. 108–269th day. Supplements.	Necrosis of liver. Number %.	Time of death. mean (days).	S.D.	270–478th day. Supplements.	Necrosis of liver. Number %.	Time of death. mean (days).	S.D.	Survived unharmed (number %).
1	11	0	0	11 (100%)*	77±29·7		—	—	—		—
2	8	Toc	0	7 (87%)†	120±8·6		—	—	—		—
3	7	0	Toc	0	—		0	7 (100%)*	65±59·5		0
4	7	Toc	Toc	0	—		0	5 (71%)‡	91±25·8		2 (29%)
5	8	0	Cys	0	—		0	8 (100%)‡	84±20·3		0
6	7	Toc	Cys	0	—		0	5 (71%)*	96±22·3		2 (29%)

S.D. = Standard deviation of the series.
† The eighth rat had post-necrotic scarring only.
* One rat showed necrosis and post-necrotic scarring.
‡ Two rats showed necrosis and post-necrotic scarring.

The female rats were raised on the stock diet and maintained on this throughout pregnancy and lactation. From the 10th day after birth until the 21st day, when they were weaned, the litter partook of this stock diet. On weaning the young rats were given the low-tocopherol moderate casein diet and divided into two sets. One set (22 rats) received a supplement of tocopherol (5 mg. per rat twice weekly); the other (26 rats) no supplement. The two sets received these diets for 107 days after weaning, then each was divided into three groups. All were then given the yeast diet, deficient in both tocopherol and thio-amino-acids. One group of each set received this alone, one of each with supplements of tocopherol and one of each with supplements of cystine.

The rats receiving the yeast diet alone, and which had had no tocopherol in the preparatory period, died of massive hepatic necrosis in an average of 77 days (S.D. $\pm$ 29·7); these on the same yeast diet, but which had received tocopherol in the preparatory period, in an average of 120 days (S.D. $\pm$ 8·6). No deaths occurred throughout the period of 162 days on the yeast diet in the groups supplemented either with cystine or tocopherol, irrespective of whether the preparatory diet had been supplemented with tocopherol or not.

From these latter four groups the supplements were then withdrawn so that the animals received the yeast diet alone. At this time the rats were 290 days old. The mean time of death for the four groups, during they subsequent 209 days on the yeast diet alone, was between 65 and 96 days, with a fairly large scatter in the time of individual deaths from hepatic necrosis. Twenty-nine per cent of the rats in the two groups which had received tocopherol in the preparatory period survived those 209 days, and their livers were without necrotic lesions. All the animals in the other two groups which had not received tocopherol in the preparatory period developed liver lesions. There was no evident difference in the length of protection afforded by the previous cystine or tocopherol during the period of 162 days on the supplemented yeast diet.

Three points appear from this experiment: First, that the tocopherol content of the preparatory diet significantly influences the survival of rats subsequently given a diet deficient both in tocopherol and thio-amino-acids; second, that the effect of this doubly deficient diet in producing massive hepatic necrosis is prevented by remedying either deficiency alone; and third, that the protective action of cystine or tocopherol when added to a diet deficient in both these substances persists for a considerable time after either is withdrawn. The further investigations reported here were designed to establish and extend the indications derived from this pilot experiment.

(b) *Experiments on rats born of tocopherol-depleted mothers.*

The previous experiment showed that rats maintained on the stock diet until weaning and then given a low tocopherol diet, died, on the average, 77 days after receiving the yeast diet. This survival time was not greatly different from that of some animals who had been maintained on the stock diet throughout the pre-experimental period. It was thought possible, therefore, that significant stores of tocopherol might be acquired during pregnancy, and from the maternal milk, when mothers were receiving the stock diet, and also, in the period between the 10th day after birth and weaning, when both mother and litter were consuming the stock diet. An attempt was made, therefore, to rear litters from tocopherol-deficient mothers, and to keep both mother and litter on this diet until the young

were weaned. By rearing mothers for a few months on the low tocopherol diet (0·5 to 1·0 mg. Toc./100 g. diet) living litters were obtained, but so many of the young died with convulsions before weaning that this method proved impracticable for the supply of animals on an adequate scale. Nevertheless when 8 animals were weaned successfully and then immediately given the yeast diet, they died on the average at the remarkably early time of 15 days (S.D.$\pm$3·1).

A compromise method was, therefore, adopted and used in all subsequent experiments. It seems that little tocopherol is transferred across the placenta or contained in the rats' milk, and that the young rat only acquires significant amounts of tocopherol from the 10th day after birth, when it begins to eat the diet available to the mother (Mason and Bryan, 1938). Breeding rats were therefore maintained on the stock diet throughout pregnancy and until the 10th day after delivery. Thereafter a tocopherol-deficient diet (25 per cent casein diet) was given. With this procedure convulsions did not occur in the young, but, as subsequent experiments show, a significant and reproducible degree of tocopherol depletion had been obtained.

(c) *Effects of tocopherol supplements in the suckling period.*

One hundred and twenty-six weanling rats were prepared in the above manner, and were given the yeast diet. These rats were the simultaneous controls for the 4 groups to be described in the next paragraph. One hundred and twenty-four died from acute massive hepatic necrosis after an average of 28 days (S.D. $\pm$10·1) on this diet. The remaining two died on the 67th and 84th days with an acute lesion superimposed upon the scars of a previous acute hepatic necrosis. This consistently early death was quite outside our previous experience. The effect of giving supplements of tocopherol in the period before weaning is described below, and the results are summarized in Table III.

When 3 mg. of tocopherol are given by mouth on alternate days to suckling rats from the 10th day until weaning and then the yeast diet is given, death from necrosis is delayed. Of 27 rats so treated 26 died in an average of 59 days (S.D.$\pm$15·8) after receiving the yeast diet, but one survived unharmed until killed on the 111th day. Supplements of tocopherol given only to the mother also delayed death. Of 25 rats from mothers, who for the last ten days before their young were weaned received 5 mg. of tocopherol daily, all died of acute necrosis in an average of 42 days (S.D.$\pm$17·6) after being given the yeast diet. This suggested an increased tocopherol content of the milk. Mixing tocopherol with food, 50 mg. daily, so that from the 10th day onwards till weaning both mother and litter received tocopherol, also delayed death. Of 48 rats in such an experiment who were given the yeast diet after weaning, 45 died in an average of 47 days (S.D.$\pm$16·5), but three survived without any lesion till the 111th day, when they were killed. Lastly, the effect of one single dose of tocopherol to weanlings who had been receiving the tocopherol-deficient diet was tested. Again protection was observed, but it did not appear to matter whether the dose was 5, 15 or 30 mg. Twenty-seven rats were given single doses immediately before the yeast diet was given. Twenty-five died in an average of 41 days (S.D.$\pm$11·1), and two survived to the 51st and 68th days respectively, when they died with acute necrosis superimposed upon a previously healed lesion.

It is evident that, by whatever means the supplementation with tocopherol was made, it conferred a significant degree of protection against the development

TABLE III.—*Effect of Tocopherol Supplements in the Suckling Period on Speed and Incidence of Death when Yeast Diet is given subsequently.*

Tocopherol supplements.	Number of animals.	Acute necrosis.				Acute necrosis and post-necrotic scarring.			Survived unharmed.		
		No.	%	Time of death. Mean (days).	Time of death. S.D.	No.	%	Time of death (days).	No.	%	Days.
0	126	124	98	28	±10·1	2	2	67, 84	0	0	—
To sucklings : 3 mg. every second day	27	26	96	59	±15·8	0	0	—	1	4	111
To mother : 5 mg. daily	25	25	100	42	±17·6	0	0	—	0	0	—
To mother and suckling : 50 mg. daily in food	48	45	94	47	±16·5	0	0	—	3	6	111
To sucklings : single dose 5–30 mg. just before weaning	27	25	93	41	±11·1	2	7	51, 68	0	0	—

Time of death is given in days after the yeast diet was started. S.D. = Standard deviation of the series.

TABLE IV.—*Effect of Protein Content of Pre-experimental Diet.*

Diet up to weaning.	Diet for 21 days after weaning.	Experimental diet.	Number of animals.	Healthy survivors after 117 days of experimental diet.		Acute massive necrosis of liver.			
				No.	%	No.	%	Time of development on experimental diet. Mean (days).	S.D.
Toc − S +	Toc − S +	Toc − S −	23	3	(13%)	20	(87%)	65	±16·6
Toc − S +	Toc + S +	Toc − S −	22	11	(50%)	10*	(46%)	..	..
Toc − S +	Toc + S −	Toc − S −	13	0	..	11†	(85%)	24	± 6·6

Toc = Tocopherol.
S = Sulphur-containing amino-acids.
Toc − S + = 25 per cent casein, low tocopherol diet.
Toc + S + = Same diet, supplemented with 120 mg. of tocopherol over 3 weeks.
Toc + S − = 7 per cent yeast protein diet supplemented with tocopherol 120 mg. over 3 weeks.
* 1 died after 105 days on experimental diet with acute necrosis superimposed upon scars of a previous necrosis.
† 1 died with acute necrosis plus previous scars at 51 days ; the other was killed at 71 days and showed post-necrotic scarring.

of acute necrosis when the yeast diet was given subsequently. The degree of protection was similar in each experiment, so that all can be grouped together. It then appears that, while the 126 animals receiving no supplement died in an average of 28 days (S.D.±10·1), the 127 rats receiving tocopherol before they were given the yeast diet died of necrosis in an average of 47 days (S.D.±17·1). Fig. 1 illustrates the distribution in time of deaths from acute liver necrosis in 249 young rats, and the effect of tocopherol supplementation during lactation.

The supplementing with tocopherol did not merely postpone deaths, it spread them over a longer period of time. Indeed some rats survived as long as 111 days without developing hepatic necrosis. This wide spreading of the period in which deaths occur is similar to that observed in animals which in the pre-experimental period have received an ordinary stock diet. This observation is of some importance, as it has been suggested that the variability in time of appearance of death in animals submitted to the same régime of depletion is inconsistent with the explanation that this type of hepatic necrosis is due to nutritional deficiency,

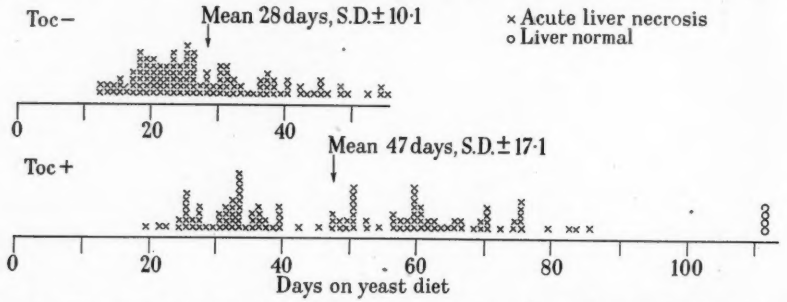

FIG. 1.—The effect on the incidence of acute liver necrosis of tocopherol supplementation during the last ten days before complete weaning. Toc − = tocopherol-free diet in preparatory period. Toc + = tocopherol-free diet in preparatory period + supplements of tocopherol.

indicating rather that some positive, perhaps infective factor is being transmitted from animal to animal.

Supplements of tocopherol during lactation did not influence the body-weight of rats when weaned (mean b.wt. was 31 g., range 20 to 55 g.), or their subsequent growth when fed on a yeast diet (*ad libitum*) immediately after weaning. The growth was very variable. Some animals lost 2 to 4 g. during the experiment and some gained more than one gramme a day, but the majority gained less. This variability in growth rate was not related to the previous tocopherol supplementation or the initial body-weight. The mean body-weight gain of 249 rats fed on yeast diet was 0·4 g. per rat per day.

There was no evident relation between the incidence of liver necrosis and the growth rate of these young animals ; but further experiments, specially devised for the purpose, would be required to exclude such a relationship with certainty.

(d) *Effect of tocopherol supplements in the preparatory period on older rats.*

Previous experience with rats raised on stock diets had indicated that, in general, the older the rat the longer the delay before hepatic necrosis developed

after the yeast diet was given. In view of the above results this finding might be due either to the older animals having a smaller need for tocopherol, or having acquired larger tocopherol stores in the longer pre-experimental period on the stock diet. To try and distinguish the effect of age, experiments were performed in which rats were raised to various ages on the low tocopherol high casein diet and then, for a fixed short period before the yeast diet was given, received a known supplement of tocopherol.

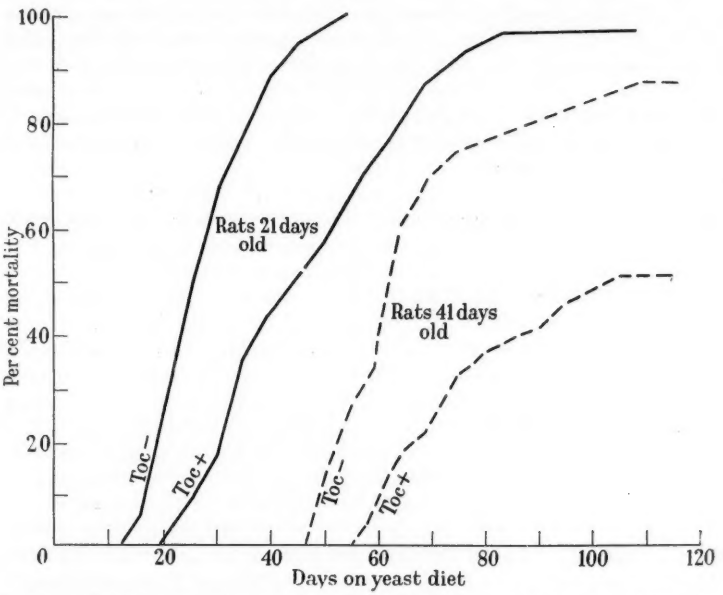

FIG. 2.—The cumulative mortality-rate of rats in relation to age and to tocopherol supplementation during the pre-experimental (preparatory) period. Toc + = previously supplemented with tocopherol. Toc − = not supplemented.

From the 10th day after birth the rats received the low tocopherol high casein diet but, for three weeks before the yeast diet was started, half the number of rats received each a supplement of 20 mg. of tocopherol a week. The first two groups of rats were 41 days old when the yeast diet was given; the weight of those which had received tocopherol supplements over the previous three weeks averaging 98 g. (S.D.$\pm$14·1), those which had not, 97 g. (S.D.$\pm$16·6). Of the 23 rats receiving no supplement, 3 (13 per cent) survived 117 days without developing hepatic necrosis: of the 22 receiving previous tocopherol supplements, 11 (50 per cent) survived unharmed, although the yeast diet was given for the whole of this time. In both groups the deaths from necrosis were spread so widely that a graphic representation of the results is more informative than a numerical one. Fig. 2 shows the cumulative mortality in these two groups plotted against time, and, for comparison, the cumulative mortality-rates for the two groups of weanling rats, one supplemented and the other unsupplemented with tocopherol,

are given. It will be seen that the 41-day-old rats survive for a longer period than the weanlings without developing hepatic necrosis when given the yeast diet, and further, that supplements of tocopherol in the pre-experimental period confer more protection in the older rats. Similar experiments were carried out with groups of rats at 71, 91 and 141 days old. In these older rats (total number 78 animals) no necrosis occurred in experiments lasting up to 150 days on the yeast diet.

Thus a marked diminution of susceptibility to dietetic hepatic necrosis and an increasing protective effect of tocopherol occurred with increasing age. It will be evident, however, that these experiments do not distinguish the effect of age from that of rate of growth. On the yeast diet growth was poor, but more so in the older animals. All lost weight (up to 30 g. in 50 days) when transferred to the yeast diet restricted to 8 g./rat/day. The loss was more marked in the heavier rats. After some time (20–50 days) the lighter animals stopped losing weight and remained stationary.

(e) *The effect of the quantity and quality of the protein in the pre-experimental diet.*

In the course of the preceding experiments evidence accumulated that the quantity and quality of the protein in the preparatory diet had also an influence on the survival of rats subsequently given a yeast diet. In the pilot experiment, first described, the low tocopherol diet used in the preparatory period contained 15 per cent of casein and 4 per cent of yeast protein. The yeast diet was given when the rats were 128 days old. Those which in the preparatory period had received supplements of tocopherol survived on the average 120 days; those receiving no supplements died in 77 days. The low tocopherol high casein diet used subsequently contained 25 per cent of casein. In the preceding section experiments were described showing that rats reared on this diet to 71, 91 and 141 days of age failed to develop hepatic necrosis when given the yeast diet for 150 days. This effect of the protein, or amino-acid, composition of the preparatory diet was further demonstrated in the experiment summarized in Table IV.

The rats were weaned at 21 days, mother and litter having had access only to the low tocopherol high casein diet during the last 11 days of suckling. They were divided into three groups, and each received one of the following diets for the ensuing 21 days: The first group continued on the low tocopherol high casein diet (Toc − S + diet); the second received that diet, but with supplements of tocopherol totalling 120 mg. per rat over three weeks (Toc + S + diet); the third the 7 per cent yeast protein diet with a similar supplement of tocopherol (Toc + S − diet). After this preparatory period the animals in all three groups were given the unsupplemented 7 per cent yeast protein diet. It will be seen from Table IV that the rats which had received the high casein diets in the preparatory period lived for long periods on the yeast diet, and longer if they had also previously received tocopherol supplements; but that the animals which had received the yeast protein diet supplemented with tocopherol in the pre-experimental period lived only for 24 days (S.D. ± 6·6) when given the yeast protein diet alone.

In a further experiment two groups of weanling rats, similarly depleted of tocopherol during suckling, were given the yeast diet during the 21 days of the preparatory period. The diet in one group (22 rats) was supplemented with

cystine (0·63 g. cystine/100 g. diet, i.e. 20 to 30 mg./rat/day) in the other with the same amount of cystine and 5 mg. of tocopherol per rat per day (total 110 mg. tocopherol). On withdrawing the supplements and giving the yeast diet alone, the group which had previously received cystine died in an average of 11 days (S.D. $\pm$ 7·8) with acute hepatic necrosis. The result in the group previously supplemented with both cystine and tocopherol was unexpected, and is unexplained. Of the 18 rats, 5 died between the 19th and 27th day of acute hepatic necrosis; the remainder died between the 4th and 28th day without necrosis. The livers of the latter were normal, or showed vacuolation of liver cells most marked in the periphery of the lobes. These vacuoles were stained yellow by Sudan III, and not red as in ordinary fatty infiltration of the liver. There was also a number of pycnotic nuclei.

It is evident from these experiments that a pre-experimental diet deficient qualitatively or quantitatively in protein will, even when the tocopherol intake is adequate, predispose to the development of massive hepatic necrosis when rats are subsequently given a low tocopherol low thio-amino-acid diet. It is also evident that, just as younger animals are more susceptible to the effects of tocopherol deficiency in the pre-experimental period, so they are more susceptible to a preliminary protein deficiency.

DISCUSSION.

The experiments reported in this paper had as their first objective an investigation of the effects of differences in the composition of the pre-experimental (preparatory) diet upon the liability of rats to develop massive hepatic necrosis when they were subsequently given a low tocopherol thio-amino-acid deficient diet. They show that this liability is increased if the preparatory diet is low in tocopherol or in protein, and that the younger the animal the more likely it is to be affected by such deficiencies.

The results obtained in the present experiments serve incidentally to underline the interdependence of tocopherol and protein, more particularly cystine, deficiency. The remarkable fact was already established that supplements of either cystine alone or tocopherol alone will completely protect against the massive hepatic necrosis which results from a diet deficient in both cystine and tocopherol. The present experiments show that the period of survival conferred by a limited course of either of these supplements to animals which later continue to take such a doubly deficient diet increases markedly with age. Similarly, at all ages tocopherol or protein deficiencies, in the preparatory period, predispose to the subsequent development of dietetic hepatic necrosis when the doubly deficient diet is given. Such clear correspondence would seem to provide a strong clue that tocopherol deficiency and protein deficiency both focus on a common point in metabolism.

At first sight it appears curious that weanlings on a low tocopherol high casein diet should readily develop massive hepatic necrosis when given the yeast diet, while older animals who have had a longer period in which to become depleted of tocopherol do not develop the lesion as readily. But it should be remembered that the low tocopherol diet is not completely free from tocopherol, and, indeed, the typical muscle lesions were seen in no animals. A small experiment (Glynn, Himsworth and Neuberger, 1945) indicates that if it had been so,

the older, and consequently more depleted animals, would have developed necrosis when deprived of thio-amino-acids at least as quickly as the young ones. Two rats had been kept from their 41st day of life to their 159th day on a diet in which nitrogen was supplied by an adequate mixture of synthetic amino-acids equivalent to 1 g. of casein daily. The rats grew well and were in excellent condition. No tocopherol supplements were given, and their companion killed on the 152nd day showed well-marked lesions of tocopherol deficiency in the voluntary muscles. The two rats were then placed on a diet in which the thio-amino-acids had been removed from the synthetic mixture. They died on the 8th and 22nd day respectively from fulminating massive hepatic necrosis, and they showed lesions of tocopherol deficiency in the voluntary muscles. The most probable explanation of the curious observation in these present experiments seems to be the following: The indications are that the requirements for tocopherol diminish with age. Throughout the period on the low tocopherol high casein diet (which, as stated, contains traces of tocopherol), the high casein content prevents death from hepatic necrosis. If rats are taken off this diet when their requirements are high, as in weanlings, and placed on the necrogenic diet, their small stores of tocopherol are rapidly used up and necrosis develops. If, however, rats are kept on this diet until their requirements are low, they will have accumulated from the traces of tocopherol in the diet a sufficient, but small amount to protect them for some considerable time after receiving the necrogenic diet.

A further point which requires explanation is that, although addition of the above amounts of cystine to diet deficient in both tocopherol and thio-amino-acids prevents the development of massive hepatic necrosis, such a supplemented diet does not confer the same degree of protection against the development of this lesion when this doubly depleted diet alone is given subsequently, as does a 25 per cent casein diet which actually supplies less sulphur-containing amino-acids. Some protection is conferred by cystine in the older animals, but it is practically without effect in young rats, even when reinforced by supplements of tocopherol. Our yeast is, however, biologically inadequate in many respects and, further, the protein content of the yeast diet was only 7 per cent as compared with 25 per cent in the casein diet. Further work is clearly necessary to determine the exact role of protein in the preparatory period in conferring resistance to the development of hepatic necrosis when the doubly deficient diet is given subsequently.

SUMMARY.

1. The composition of the diet taken by rats before they are given a low tocopherol low thio-amino-acid diet influences conspicuously the speed of development and incidence of the massive hepatic necrosis which develops under this régime.

2. Deficiency of tocopherol and qualitative and quantitative deficiency of protein in the pre-experimental diet predispose, and supplementation with tocopherol or adequate good protein reduce the liability, to the development of massive hepatic necrosis when the low tocopherol low thio-amino-acid diet is given.

3. Supplements of tocopherol or of cystine to animals on a low tocopherol low thio-amino-acid diet not only confer protection against the development of

hepatic necrosis while they are being given, but also give protection for some time afterwards.

4. The liability to develop massive hepatic necrosis decreases with age. The protective action of tocopherol, or good quality protein, such as casein, given in the preliminary period before the low tocopherol low thio-amino-acid régime, increases rapidly with age, so that older animals which have received a rich stock diet may fail to develop the hepatic lesion when they are subsequently given a diet deficient in tocopherol and thio-amino-acids. A similar protective action of supplements of cystine given in the same period can be shown in older animals, but is hardly demonstrable in weaning rats.

5. This influence of the composition of the preliminary diet upon the subsequent development of dietetic necrosis of the liver goes far to explain the discrepancies between the results of different workers in this field.

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REFERENCES.

Glynn, L. E., Himsworth, H. P., and Neuberger, A.—(1945) *Brit. J. exp. Path.*, **26**, 326.

György, P.—(1947) ' Liver Injury,' *Trans. of the 6th Conf. Josiah Macy Jr. Foundation, N.Y.*, p. 67.—(1948) ' Biological Antioxidants,' *Trans. of the 3rd Conf. Josiah Macy Jr. Foundation, N.Y.*, p. 71.

Himsworth, H. P., and Lindan, O.—(1949) *Nature*, **163**, 30.

Lindan, O., and Work, E.—(1950) *Biochem. J.* (in press).

Mason, K. E., and Bryan, W. L.—(1938) *Ibid.*, **32**, 1785.

Tosic, I., and Moore, T.—(1945) *Ibid.*, **39**, 498.

REGENERATION IN THE SUBMAXILLARY GLAND OF THE RAT.

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THE mechanism of regeneration in salivary tissue has not been investigated since the researches of Podwyssozki (1887) and Ribbert (1894), who studied the problem in the rabbit. At that time, the tubulo-racemose structure of the submaxillary gland had not been described (Müller, 1895 ; Cohoe, 1907) so that the place of the tubular part of the gland in the regenerative process was not appreciated.

METHODS.

The experiments were performed on the submaxillary glands of male albino rats. The rats were selected for uniformity in body weight, and weighed either 200 or 250 g. approximately. Two experiments were performed, the first qualitative, the second quantitative. For the first experiment, the rats were divided into groups of six. They were operated on under open ether anaesthesia with full aseptic precautions. Through a midline incision in the neck, one submaxillary gland was exposed, and separated from the major sublingual gland, which is enclosed in the same capsule. About five-sixths of the gland was removed from half the animals in the group. The main vessels and duct were ligated with 43 S.W.G. stainless steel wire, but the cut surface of the gland, which bled only slightly, was not ligated. The other half of the group served as controls. These animals underwent mobilization of the gland, which was then allowed to fall back into its bed. Each group was killed at intervals between 1 day and 3 months after operation.

In the second experiment, three groups of 6 animals were used. One group was killed, and both glands weighed, the major sublingual glands being included. This provided the mean weight of one gland. The second group underwent removal of one gland and half the other, and were allowed to survive for 3 weeks. In this group the divided gland was cut across with scissors, and no ligature was used, haemorrhage being controlled by temporary pressure. The third group served as controls. These underwent mobilization of one gland and half the other, and were also killed after 3 weeks. The last two groups were pair-fed before and after operation in order to maintain their weights equal as far as possible. All animals were fed on M.R.C. rat cubes and water.

At the end of the experiments, the animals were killed with ether, and the remaining glands and portions of glands were dissected out and weighed. The animals in the first experiment received an intraperitoneal injection of colchicine (2 mg./kg.) 8 hours before death in order to arrest mitosis, and they were all

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killed at the same time of day. All the glands were fixed in 10 per cent formol-saline, and paraffin sections prepared for staining with Ehrlich's haematoxylin and eosin or Altmann's acid fuchsin and picric acid. The latter stain is useful for identifying the granules in the tubular portion of the gland.

In another group of 6 animals, the parotid, major sublingual and submaxillary glands were removed in two-stage operations, leaving only the minor sublingual glands as functioning salivary tissue. These rats were killed 3 months after the second operation, and the minor sublingual glands were dissected out and weighed. The tissues round the mouth and jaws were carefully examined for the presence of regenerated salivary tissue.

RESULTS.

Qualitative changes.—No macroscopic evidence of regenerated tissue could be seen. Microscopical examination of specimens removed one day after operation, showed marked degeneration. In some preparations all the acinar and duct cells in whole lobules had undergone cloudy swelling and fatty degeneration. In others, a fringe of normal acini persisted at the edges (Fig. 1). The cells which had not undergone degeneration appeared normal apart from an increased separation due to oedema. Contrary to the findings of Podwyssozki (1887) no mitoses were noted. Some of the smaller veins were thrombosed. After 3 days granulation tissue could be seen growing into the cut edge of the remaining portion of gland. The acini in the adjacent gland showed a moderate number of mitoses, but the most striking change was in the wall of the main duct. This was now 3 to 4 cells thick, instead of the normal single layer, and there were numerous mitoses amongst these cells (Fig. 2). A control gland which had been slightly damaged during mobilization also showed many acinar mitoses.

After 5 days the hyperplasia of the lining cells of the main ducts was still pronounced and mitoses were numerous. Branching ducts could now be seen growing into the granulation tissue along the cut edge, and in places these were proliferating to form acini. The process of formation of new acini from the old ones by division could still be seen in progress close to the line of section (Fig. 3). The nuclei of the new cells were larger than normal. The new acini were small and darker-staining than the mature ones, the cytoplasm staining deep red with eosin. Some of the new acini had a small lumen. The acini at the ends of the proliferating ducts were small, and composed of 2 to 4 cells. An early stage of duct proliferation is shown in Fig. 4. Areas in which new small acini were developing from ducts were sometimes seen in the normal gland away from the cut edge, and also in the controls, in a few cases (Fig. 7). These presumably represent compensatory hypertrophy or normal growth of the gland.

At the end of a week the proliferation of ducts and their differentiation into acini was still more marked and division of acini was taking place. One control gland at this stage showed duct proliferation with many mitoses in a small area at the edge in which haemorrhage had occurred during mobilization (Fig. 8).

After this time, both early and late stages of regeneration could be seen in the same sections. This is not surprising since regeneration would be expected to show first in the least damaged areas and later in areas which have suffered more degeneration.

At 2 weeks, what appeared to be a completely regenerated lobule of gland was seen in one preparation (Fig. 9). In a section stained with Altmann's acid

fuchsin, the tubular part of the gland could just be recognised in the new lobule in the form of occasional cells containing zymogen granules. The nuclei in the tubular part were fatter, paler and more numerous than normal and the granules in the cytoplasm less eosinophilic and smaller.

In one preparation obtained at 3 weeks, a newly formed gland composed of at least 6 lobules was seen (Fig. 11). There was a fringe of normal gland along one edge. The main duct was bent into a U-shape. The connective tissue in the new gland was not increased, i.e. the gland was formed entirely of new ducts and acini. The main ducts, faintly eosinophil, and lined with columnar epithelium, were breaking up into fine eosinophil ducts, lined by low columnar epithelium, which had much smaller nuclei. From these the acini budded off. The acini were at first dark red and solid. Later they enlarged, became round and had a small lumen. When they had grown to full size, they became irregular from the pressure of adjacent acini, and the lumen disappeared. The distal parts of the ducts at this stage had not developed cytoplasmic granules and still had the appearance of ducts and not that of the tubular part of the gland. This was confirmed with Altmann's stain. The bending of the duct seen in this preparation is the cause of obstructive changes which were seen in some specimens and which prevented regeneration in them.

Another regenerated lobe was seen in a specimen at 4 weeks (Fig. 12). This showed new acini in all stages of development. The younger ones were small and stained pink with eosin. They had relatively large but not very dense nuclei. The mature ones were larger, and their cytoplasm was slightly basophil. Occasional tubular areas could be distinguished in this specimen.

In later specimens complete regeneration was difficult to identify. Many apparently normal lobes were noted but these may have survived from the time of operation. Three features were found useful in determining whether a lobe had regenerated; separation of all the acini by oedema, crowding of the ducts suggesting that they had regrown parallel to one another, and absence or sparseness of the tubular part.

These features were all in evidence at 5, 6 and 8 weeks. In one 8-week specimen there were two newly-formed lobules, one of which possessed a tubular part. In this specimen also a long terminal duct showed acini budding off it (Fig. 14). Fig. 16 depicts a new lobule after ten weeks with its main duct entering it. The acini still stain slightly darker than normal and the tubular part is only slightly developed.

Quantitative evidence of regeneration.—The results obtained are summarized in Table I. It will be seen that the submaxillary glands of normal 200 g. rats may be expected to increase in weight by 26·5 per cent in 3 weeks. The mean increase in weight of the portions of gland left after partial removal was approximately 22 per cent, suggesting that regeneration does not occur. But of the 6 animals used, the glands of 3 failed to show any increase in weight during the 3 week period of survival. The glands of the other 3 animals increased in weight by 33, 41 and 47 per cent, a significantly greater figure than was found in the controls. The reasons for this discrepancy will be discussed later.

When the animals which had lost all the salivary tissue except the minor sublingual glands were killed, no evidence of regeneration was apparent. The minor sublingual glands were not increased in size, and no other salivary tissue could be found. This suggests that there is no stimulus to regeneration of

TABLE I.

Group.	Number of rats in group.	Mean body wt. at operation (g.)	Mean wt. L. gland (g.)	Mean wt. part of R. gland removed (g.)	Mean wt. remaining R. gland (g.)	Mean body wt. 3 weeks after operation (g.)	Mean wt. R. gland 3 weeks after operation (g.)	Mean wt. L. gland 3 weeks after operation (g.)
1. Removal of L. gland and half of R. gland from 200 g. rats	6	207	210·8	112	98·8	248·7	121·5	..
2. Mobilization of L. gland and half of R. gland from 200 g. rats	6	208·5	..	..	..	257	245·4	233·8
3. Normal 200 g. rats	6	208·8	190	194		..	..	..

Body weight and weights of submaxillary glands before and after removal of one gland and half the other.

salivary tissue, for which the probable reason is that the saliva is not essential. However, it was noticed that when these animals were feeding, they found it necessary to take up a position beneath a water bottle and to drink copiously after each bite of a rat cube, presumably to moisten the food.

DISCUSSION.

Although definite quantitative evidence of regeneration in the submaxillary gland was not obtained, the microscopical findings leave no doubt that regeneration both of acini and ducts does occur, although it was not found in every gland examined. There are several reasons for this. Since five-sixths of the gland was removed, the remaining portion was very small. In spite of care in operating in order to avoid damage to the vessels and nerves, venous thrombosis was observed in some cases and the vascular damage may have resulted in ischaemia of the remaining portion of gland so that it could not regenerate. In other cases, the duct had become angulated, causing obstruction (Fig. 11). When obstruction occurred, the ductules dilated, and the acini disappeared, and finally infection of the duct supervened. Since it appears from this investigation that the most important sources of regeneration are the cut ends of the ducts, the amount of regeneration would depend partly on the number of ducts divided. In the first experiment, the gland was divided near the proximal end where its cross-section is small. The number of divided ducts from which regeneration could occur was therefore not as great as it would have been if the division had been performed at the site of the maximum cross-section.

The early changes found are similar to those described by Podwyssozki (1887), except that mitoses were not in evidence 24 hours after operation. Specimens were not examined at 48 hours, but mitoses were numerous after 3 days. Acinar mitoses were not found after 1 week, but the growth of newly formed ducts had commenced after 5 days. According to Podwyssozki (1887) these ducts mostly undergo a "regressive metamorphosis," with the formation of giant-cell conglomerations, or cysts and masses of fat droplets, only the smallest ducts being converted into new glandular acini. No regressive changes were found in my material and all the newly formed ducts appeared to develop acini at their terminations.

Ribbert (1894) was able to recognize naked-eye the regenerated areas after

removing half the submaxillary gland in the rabbit. The failure to do so in my experiment was due to the small size of the regenerated areas. The microscopical findings, however, are similar to those of Ribbert (1894), i.e. regeneration of acini occurs from the growth of large and small ducts. In my investigation it was possible to study regeneration in the tubular part of the gland. It was found that the tubular part did not appear to give rise to acini to any great extent or, if it did, the process was associated with a loss of the specific appearance and granules of the tubular cell so that it could not be distinguished from a large duct cell. Moreover, when a lobule regenerated, the regeneration of the tubular part was slow, slight and incomplete. Fig. 10 and 13 show new gland lobules stained with Altmann's acid fuchsin. The dark-staining areas are for the most part young acini, which take up the acid fuchsin, and not the fuchsinophil granules of the tubular part.

The small lumen in the new acini, on which Ribbert (1894) laid great stress as showing that the acini were newly-formed and had originated from ducts, was not seen in the earliest, dark-staining acini but only in slightly later, larger ones. The original acinar bud from the duct is solid, and the lumen only appears when the acinar cells have multiplied. It seems likely that it is not a remnant of the duct lumen, but a newly formed ductule, which is at first relatively large but rapidly narrows down to the normal size, when it is no longer visible.

The formation of new acini in the substance of the gland away from the cut edge may be regarded as compensatory hypertrophy rather than as regeneration. Ribbert (1894) concluded that the submaxillary gland of the rabbit showed only a slight tendency to hypertrophy, but this phenomenon occurred fairly often in the glands of my rats. It might be argued that this vitiates any positive results which were found in the quantitative experiment since the opposite gland was

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EXPLANATION OF PLATES.

FIG. 1.—Two lobules, one of which shows extensive cloudy swelling and fatty degeneration. 1 day. Haematoxylin and eosin. × 55.

FIG. 2.—Hyperplasia of lining cells of duct. 3 days. Haematoxylin and eosin. × 190.

FIG. 3.—Acinar mitoses. 5 days. Haematoxylin and eosin. × 535.

FIG. 4.—Early stage of duct proliferation. 5 days. Haematoxylin and eosin. × 250.

FIG. 5.—Newly-formed acini and ducts. 5 days. The new acini are above and to the right. Haematoxylin and eosin. × 190.

FIG. 6.—New acini forming from ducts. 5 days. Haematoxylin and eosin. × 250.

FIG. 7.—New acini forming away from cut edge. 5 days. Haematoxylin and eosin. × 220.

FIG. 8.—Control gland. Duct proliferation at edge in area of haemorrhage. 1 week. Haematoxylin and eosin. × 190.

FIG. 9.—New lobule. 2 weeks. Haematoxylin and eosin. × 40.

FIG. 10.—New lobule. 2 weeks. Altmann's acid fuchsin. The dark areas are young acini and not tubular parts. × 27.

FIG. 11.—New gland. 3 weeks. Fringe of normal gland above. The bending of the duct is clearly seen. Haematoxylin and eosin. × 18.

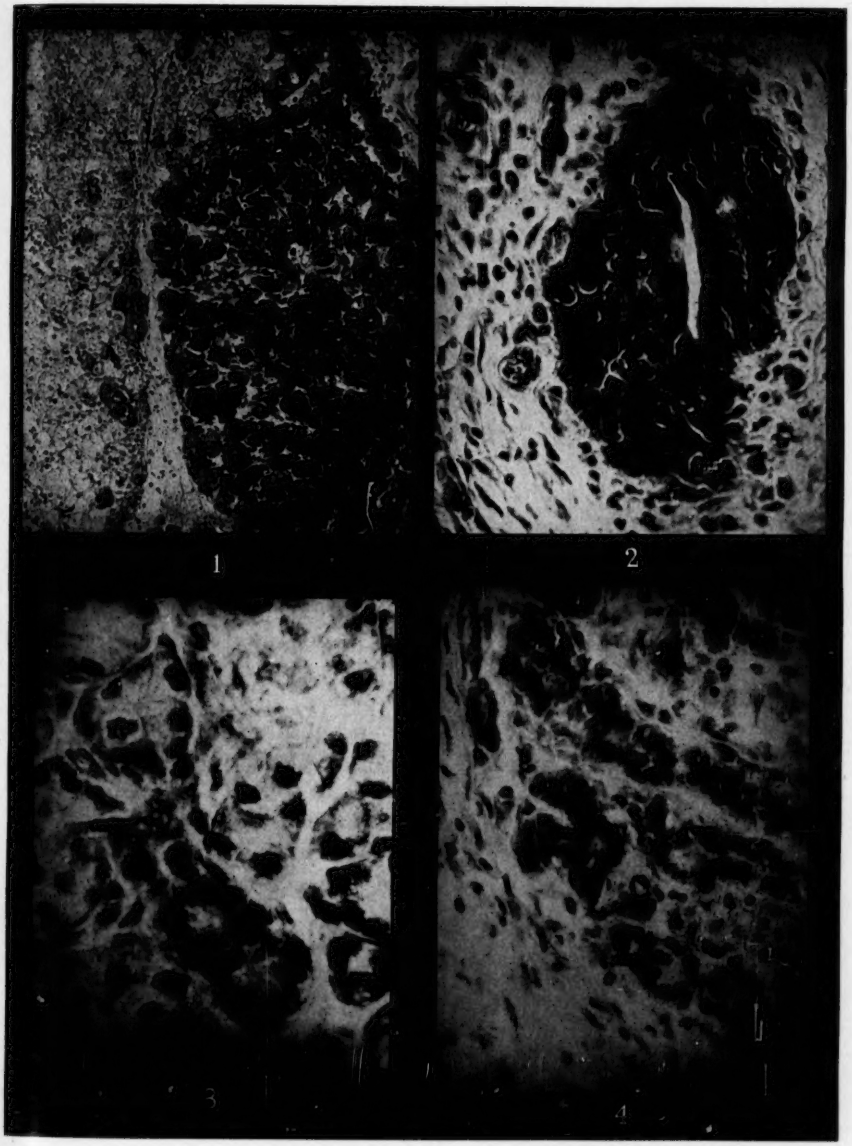
FIG. 12.—Regenerated lobule. 4 weeks. Haematoxylin and eosin. × 13.

FIG. 13.—Same as Fig. 12. Altmann's acid fuchsin. Note the sparseness of the tubular part. × 13.

FIG. 14.—Budding duct. 8 weeks. Haematoxylin and eosin. × 115.

FIG. 15.—New lobule. 8 weeks. Haematoxylin and eosin. × 78.

FIG. 16.—Duct entering new lobule. 10 weeks. Haematoxylin and eosin. × 25.



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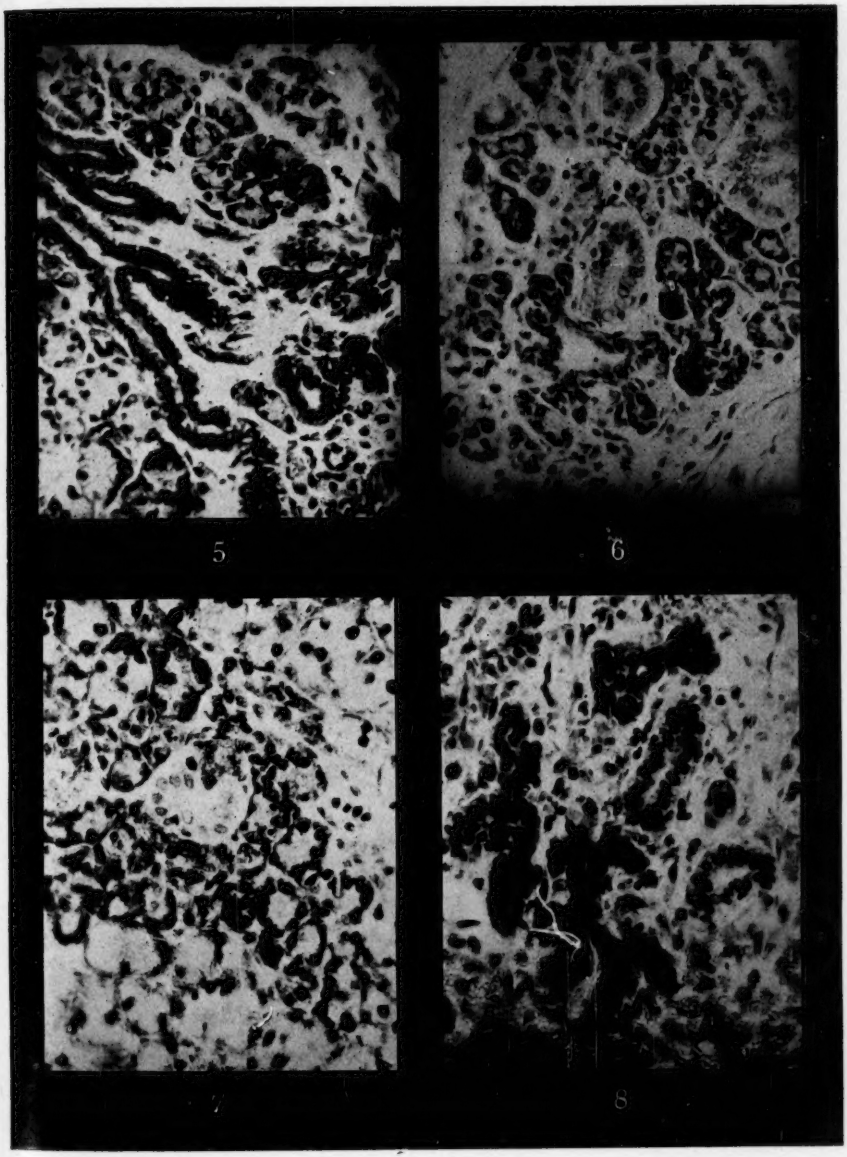

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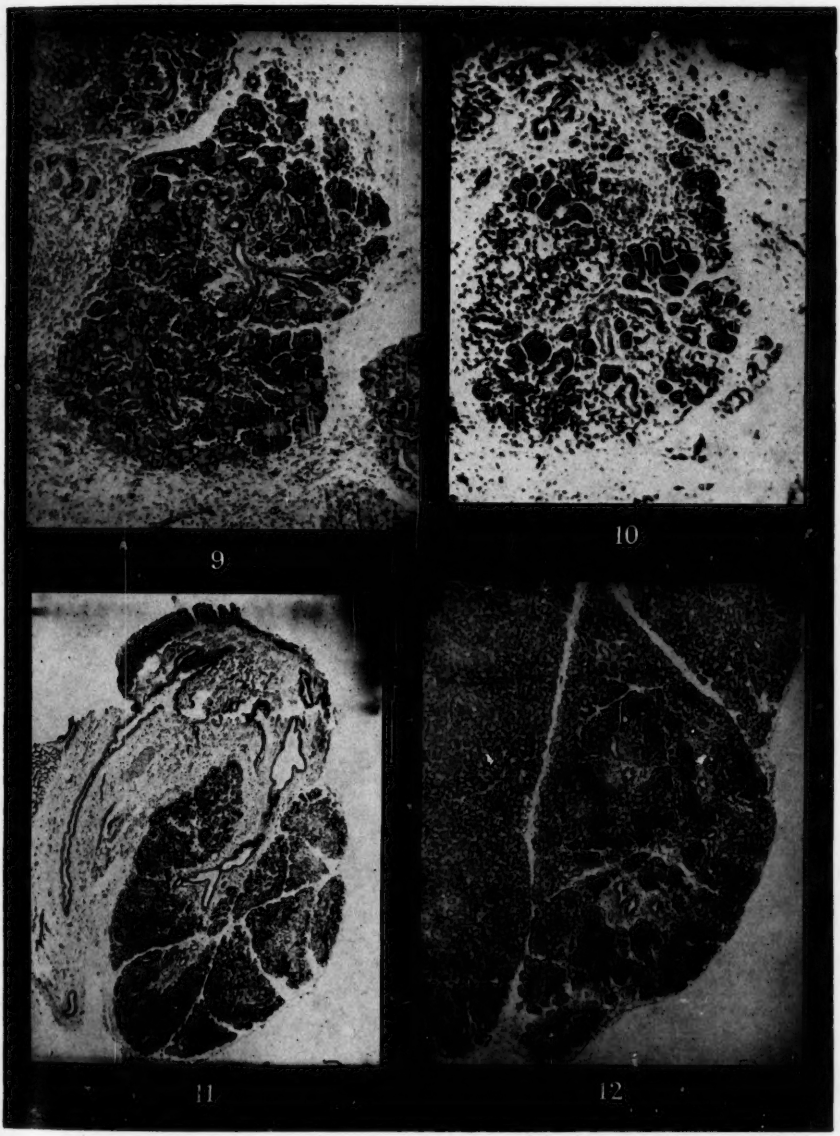

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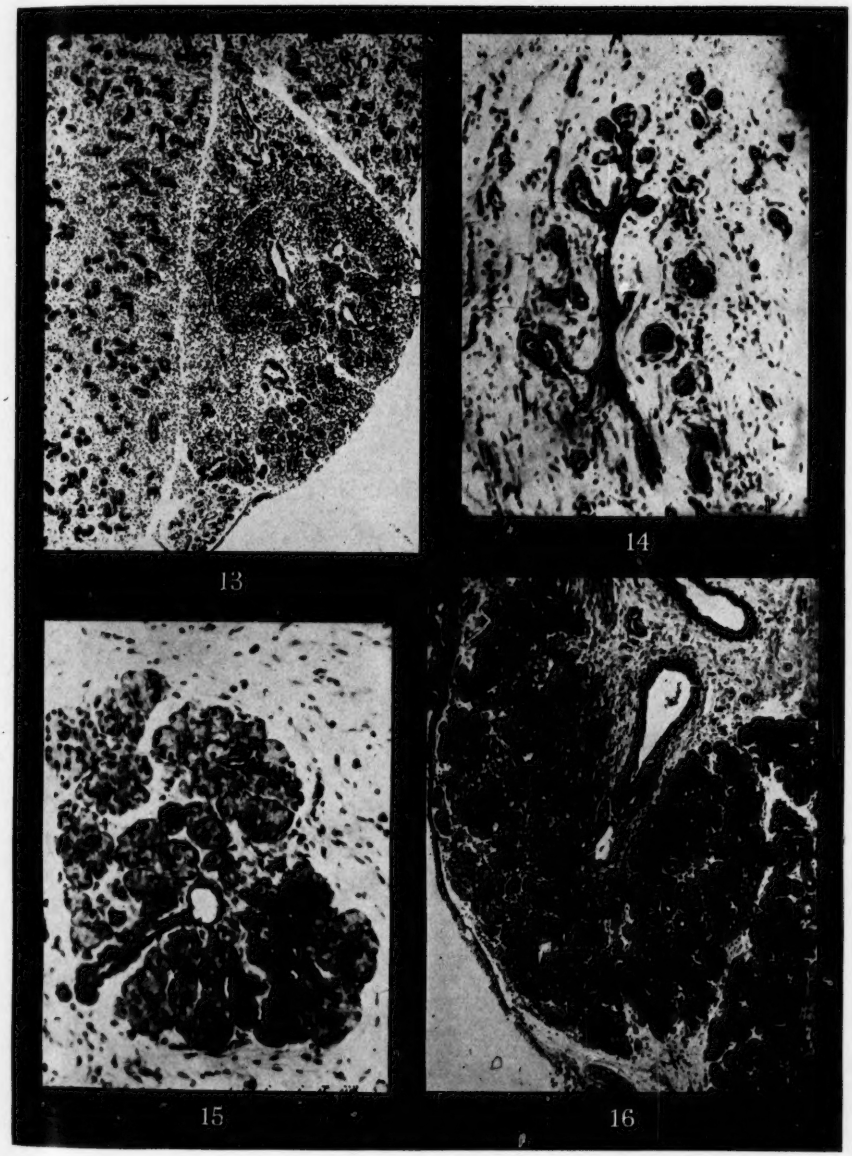

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removed. In fact, removal of glandular tissue does not appear to be a stimulus to hypertrophy, but injury to the gland does initiate regeneration, although there is no proof that the amount depends of the degree of injury. However, too fine a distinction between regeneration and compensatory hypertrophy cannot be drawn, since they both represent the same process.

The failure of regeneration after total extirpation of the major sublingual, submaxillary and parotid glands is surprising, but is in conformity with the findings of Schafer and Moore (1895–6) in the dog.

SUMMARY.

The mechanism of regeneration in the submaxillary gland of the rat after partial excision was investigated.

During the first week regeneration occurs as a result of division of pre-existing acini.

After the first week, ducts proliferate and branch in the granulation tissue and acini develop on their terminations.

The tubular part of the gland regenerates incompletely.

I wish to express my gratitude to Professor G. R. Cameron, F.R.S., for suggesting this investigation and for his guidance and helpful criticism.

REFERENCES.

Cohoe, B. A.—(1907) *Amer. J. Anat.*, **6**, 167.
Müller, E.—(1895) *Arch. mikr. Anat.*, **45**, 463.
Podwyssozki, W.—(1887) *Beitr. path. Anat.*, **2**, 19.
Ribbert, H..—(1894) *Arch. Entw. Mech. Org.*, **1**, 70.
Schafer, E. A., and Moore, B.—(1895–6) *J. Physiol.*, **19**, xiii.

PROLONGATION OF SURVIVAL OF SKIN HOMOTRANSPLANTS IN THE RABBIT BY IRRADIATION OF THE HOST.

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THE researches of Medawar on transplantation of the skin in the rabbit have done three main things. First, they have provided (Medawar, 1944, 1945) a relatively simple semi-quantitative method for the study of the mechanism of resistance to homoplastic grafts. Second, they have shown (Medawar, 1944, 1946*a*, 1948*a*) that the destruction of homotransplants has many of the characters of an immune response. Third, they have shown that if this is an immune response, it is widely different, notably in the lack of demonstrable circulating antibody (Medawar, 1946*b*, 1948*b*), from that to " ordinary " antigenic material such as bacteria.

Our own interest in this field arose out of the experiments of one of us (W. J. D.) on transplantation of the kidney. It was desired to find a means of prolonging the survival of an organ transplanted into another animal of the same species. The technical difficulties of kidney transplantation rendered any direct. attack on this problem a most formidable one, and it seemed worth while to use the much more amenable skin as a test object for the application of any procedures which might be thought hopeful. Medawar's work supplied not only the technique for this but also an obvious suggestion (derivable also from earlier work on tumour transplantation) of the type of agent likely to be worth exploring. If an immune reaction is involved, then procedures known to suppress immune reactions must be tried. Of these, the easiest to handle is irradiation, which has been shown many times (Hektoen, 1915, 1918, 1920 ; Murphy and Sturm, 1925 ; Craddock and Lawrence, 1948) to be capable of suppressing antibody production to bacteria.

Our earlier experiments consisted largely of a repetition of Medawar's, designed chiefly to establish a baseline for further work, but also with the object of confirming two of the most vital of his results : namely, that a second transplant from the same donor to the same host dies more quickly than the first, and that a large graft dies more quickly than a small one. Our experiments do in general confirm both these points, but there is a good deal of individual variation and we have not yet accumulated enough data to make a convincing case. We propose therefore to confine ourselves in this communication to the more clear-cut results of our experiments on the effect of irradiation with X-rays, using the data of our earlier experiments only in so far as they are necessary as controls.

* Working at the Buckston Browne Research Farm as a Leverhulme Research Scholar.

TECHNIQUE.

Transplantation.

Medawar's technique (1944) has been used almost unaltered. Briefly it consists in dissecting epidermis and dermis from a large rectangular area of the side of the thorax of a rabbit, placing on it several (usually eight) small pieces of skin taken from elsewhere on the same rabbit (autotransplant) or from a different rabbit (homotransplant), and covering the whole with an occlusive dressing of which the most important element is a light plaster bandage encircling the thorax. At intervals of (usually) 4 days the bandage is removed and one of the small grafts is removed for histological examination.

Two minor modifications of the original technique may be mentioned. First, plain sterile vaseline smeared thickly on gauze is better than tulle-gras. Second, rather than cut a number of separate grafts by the method used for taking pinch

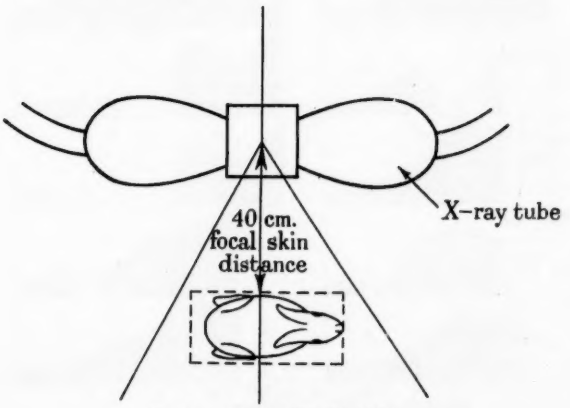


FIG. 1.—Irradiation of rabbit.

grafts in human surgery, it is simpler to cut a single strip, 5–10 mm. wide, of the clipped donor skin, to trim it in length till its weight is that of the skin dosage required, and then to divide the strip transversely into eight. (Dosages of 0·45 or 0·9 g. of skin were used in all the experiments recorded here : we have avoided complicating the description by consideration of the dosages used, as it does not in any way affect the conclusions drawn in this paper.)

Irradiation.

The arrangement for irradiating the rabbits is shown in Fig. 1. The X-ray tube was run at 190 kV., 4 mA., with the beam filtered through 0·8 mm. copper plus 0·5 mm. aluminium. The rabbit was placed in a small cage in which it could not readily turn round, and was irradiated for 8 minutes through one flank at a focal skin distance of 40 cm.; the cage was then turned round and the same length of irradiation given through the other flank. The distribution of dose through the rabbit along the axis of a single X-ray beam of the given quality is shown in Fig. 2. By irradiating through both flanks, it is possible to achieve a relatively uniform dose throughout the body of the rabbit. Measurements of

the X-ray intensity at the skin of the rabbit were made, using small calibrated condenser ionization chambers, and it is estimated that errors in these dose measurements did not exceed 2 per cent. A measurement of the dose in air at the same point but without the rabbit was 11 per cent lower, the difference being due to back-scattered radiation from the rabbit. The rabbits were not all of the same breadth from flank to flank, and for this reason the average dose of 250 roentgens which we quote is subject to an error of $\pm$ 10 per cent.

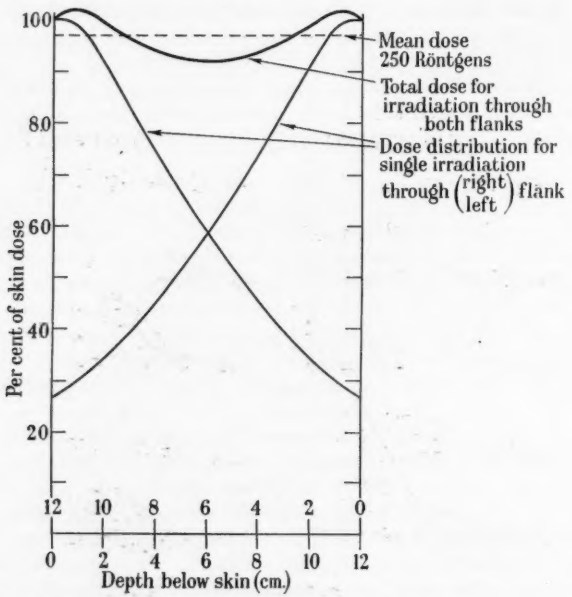


FIG. 2.—Relation of depth beneath skin to dosage, showing how the summation of irradiation for two sides can be used to produce a nearly uniform tissue dose to the whole rabbit.

A similar method, based on similar reasoning, appears to have been used by many American workers for the atomic energy projects (Hagen, Jacobson, Murray and Lear, 1949) but our method was in use before details of this were available to us.

Method of Recording Results.

The graphs which we have found the most satisfactory method of recording our results are, we hope, adequately explained by the legend to Fig. 3. The thickness of the epidermis has been recorded as a number of cells (i.e. of distinct nucleated cells, neglecting the stratum coreum). This is easier than measuring the thickness in μ, and sufficient for the purpose. The process of hyperplasia is so regular that so long as the epidermis remains healthy any two specimens which are the same number of cells thick are almost indistinguishable in such matters as stage of keratinization and differentiation and in hair follicle changes. There is some scope for subjective variation in counting cell layers, but the

specimens illustrated in Fig. 8–13 should suffice to indicate the standards used. "Healthy" epidermis is marked in the graphs by black shading. Epidermis which is abnormal but still probably alive is marked by diagonal shading, and epidermis still present but probably dead unshaded. Loss of cell outlines and uniform nuclear pyknosis are the criteria most relied on for the sometimes difficult distinction between the two latter stages. It will be seen that in many cases the final death of the grafts has been recorded on naked eye examination alone. This has only been done when all the remaining grafts simultaneously shrivelled and blackened in an unmistakable fashion.

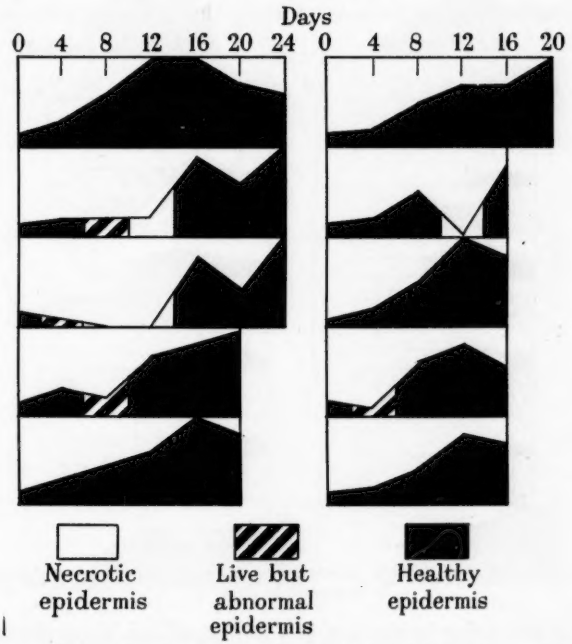


FIG. 3.—Autotransplants. Graphs showing the survival and hyperplasia of the epidermis in ten rabbits. The length of each rectangle indicates the duration of histological control, and the height the thickness of the epidermis. The full height of each rectangle indicates a thickness of ten cells; thicknesses over ten cells are shown as ten cells. The graph line, therefore, represents the course of hyperplasia in each experiment, apart from interruptions due to the accidental death of, or damage to, individual skin fragments.

RESULTS.

Non-irradiated controls.

Fig. 3 shows the results to be expected in autotransplants. Death of single grafts, due apparently to minor technical flaws interfering mechanically with primary healing, produces irregularities in some of the graphs; this forms a source of error in the method only to be avoided by using sufficient numbers of rabbits to reduce the chance of their affecting the overall picture. The general trend is clear enough. The epidermis has already begun to thicken by the 4th day,

and continues to do so with increasing speed to about the 16th day, after which it regresses back to normal. The thickening involves both mitotic multiplication of the cells of the epidermis and an increasing differentiation, so that the epidermis rapidly assumes a stratification similar to that of human skin (Fig. 9). The histology of the process, which also involves the hair follicles, has been described in detail by Medawar (1944). It is a non-specific response to trauma which can be excited in many ways, by shaving or mild chemical irritation, for instance, and it has no relationship to the transplantation as such. Its importance in the present connection is very simply this, that it involves active multiplication of the cells and is thus a very sensitive index of their " health." Under all circumstances that we have studied, the first detectable indication that

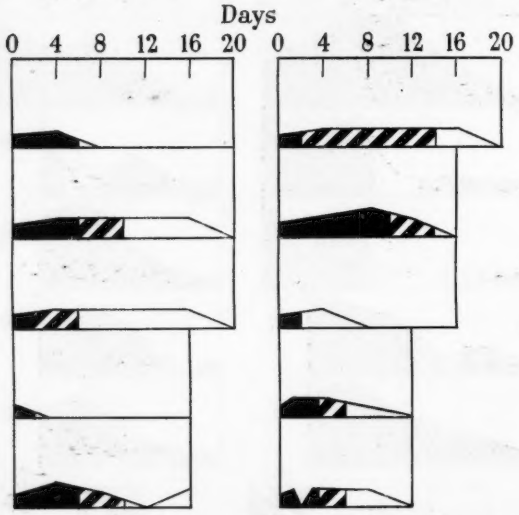


FIG. 4.—Homotransplants. Ten typical examples showing the limited hyperplasia and survival of homotransplants.

the epidermis is not going to survive is a failure of this hyperplasia. Its occurrence at what for the purpose of the experiments is a favourable time seems to be simply a lucky accident, and if the epidermis survives for longer than the period of active hyperplasia (that is to say, for about 16 days) the process ceases to be available as a measure of health of the cells. The important things to note in the autotransplant series are the initial hyperplasia in all grafts (interrupted only by the occasional accidental death of single pieces of skin) and the survival of all remaining grafts at the end of the experimental period.

The homotransplant series (Fig. 4 and 10) stands in marked contrast to this. None have survived as long as 16 days, and most are dead within 8 days. It is noticeable that even in those cases in which the epithelium has survived long enough for the hyperplasia to declare itself, it is right from the start never as steep as that of the autotransplant. The general significance of this we do not propose to discuss in the present paper, but its occurrence must be contrasted with the condition in the irradiated series.

Irradiated homotransplants.

The results in sixteen rabbits given 250 r on the day before transplantation are seen in Fig. 5. The degree of survival of the grafts varies considerably, but it is in no case less good than the average performance of non-irradiated grafts, and in seven of the sixteen the whole behaviour of the graft for the period of

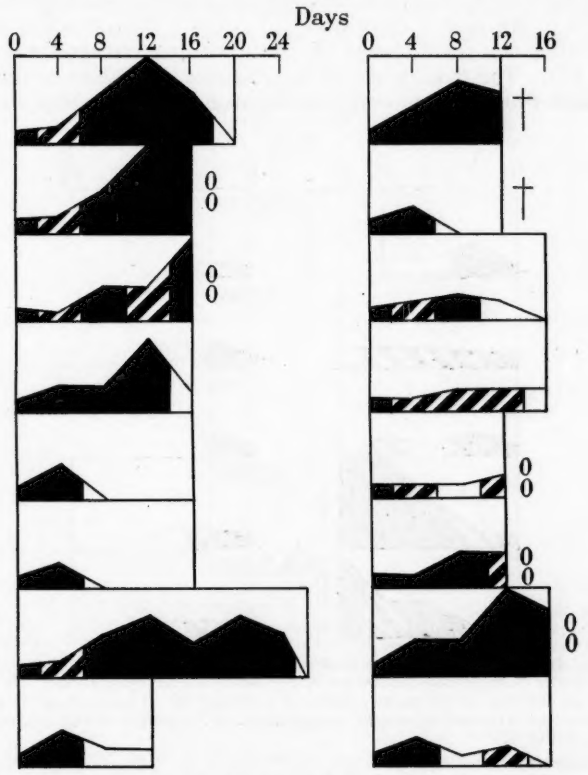


FIG. 5.—Homotransplants. Not irradiated. Each of these sixteen rabbits received 460 r before receiving the homotransplant. Contrast with Fig. 4 shows the greatly improved survival. The sign + indicates death of the rabbit. In cases where the last specimen examined histologically was alive, the sign $\overset{0}{0}$ has been used to show that at the next examination death of all remaining grafts was so obvious to the naked eye that no further material was taken.

hyperplasia is that of autografts. Four cases began with a rate of hyperplasia better than average for non-irradiated homotransplants but were dead by the 8th day ; five survived for periods of 8 to 16 days with only moderate hyperplasia and earlier death of some members of each set of grafts. One rabbit died on the 12th day, with the grafts in the full tide of hyperplasia. Of the six rabbits which survived with a full degree of epidermal hyperplasia, in one the epidermis died

before the 16th day, in four the epidermis died rather suddenly between the 16th and 20th days and in one it died between the 24th and 26th.

So far as naked eye appearances are concerned, in the better examples the grafts had for the period of their survival all the appearances of autografts; there was even a suggestion that the first take of the grafts was more satisfactory after irradiation. The final death was a sudden shrivelling up of the grafts, a graft apparently healthy at one inspection being dry, black and loose when next looked at 4, or in one case 2, days later.

Histologically, the resemblance of the grafts to autografts was confirmed (Fig. 11, 12, 13). The five (or six, if one includes one rabbit dying on the 12th day) most successful graftings were indistinguishable in any way from autografts

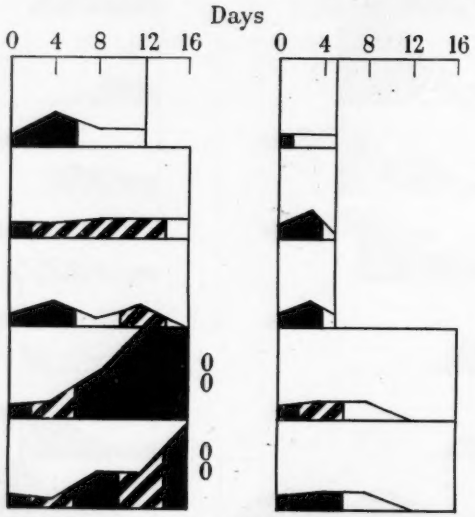


FIG. 6.—Serial homotransplants: Irradiation before the first set. The left-hand column shows the results of homotransplantation in five rabbits after irradiation. The right-hand column shows the fate in the same rabbits of a second set of transplants from the same donors. Survival of the second sets is not prolonged. (The graphs of the left-hand column appear also in Fig. 5.)

of the same age. The sequence of epidermal and hair follicle changes was exactly reproduced. Cellular infiltration was frequent, but quite irregular in its incidence, and not different in distribution or cell type from that seen in many of the autotransplants.

We have also obtained some results suggesting that irradiation of the donor improves survival of homotransplants, but our experiments in this direction are not completed and we will defer consideration of this unexpected result to a later paper.

Irradiated serial transplants.

In the rabbits of Fig. 6 a first set of homotransplants had been applied after the usual irradiation, and in all of them irradiation had produced some

improvement in epidermal survival. Approximately a week after death of the first grafts a second set from the same donor has been transplanted by the same technique to the opposite flank; but this time no radiation has been given. The second set grafts die as rapidly as usual: irradiation before the first sets has had no persistent effect on the second sets.

In the rabbits shown in Fig. 7, two sets of transplants have been made from the same donor, and irradiation has been given before the second grafting only. Under these circumstances irradiation does not prolong the survival of the grafts.

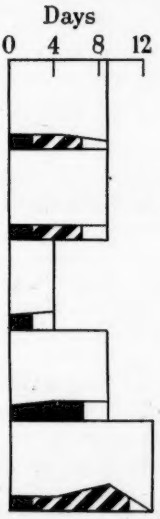


FIG. 7.—Serial homotransplants: Irradiation before the second set. Each of these five rabbits had received a first set of homotransplants without irradiation, and these died in the ordinary way. They were irradiated, and a second set applied again from the same donors. Irradiation fails under these circumstances to prolong survival of the grafts.

DISCUSSION.

The results presented in Fig. 5 seem to us inescapable evidence that it is possible to prolong the survival of homotransplanted skin by total body irradiation with X-rays. The prospect of practical application of this remains remote: the rabbit, with an MLD 50/30 days of about 800 r (Bloom, 1948, Hagen *et al*, 1949) is exceptionally resistant to total body irradiation of this type, and it is certain that doses of the order we have used would never be considered justifiable for human patients, especially as candidates for homografts are usually already in poor general condition. They indicate, however, that the homograft reaction is modifiable, and offer the hope that some effective but less drastic procedure may be discovered. There is little hope of achieving permanent survival of a homograft, but even moderate prolongation of temporary survival could in many circumstances be valuable.

From the theoretical standpoint the first importance of these results is their confirmation of the immune reaction basis of the homotransplant reaction. The

effects of irradiation on the whole body are complex, and it is by no means certain that no other mechanism is involved, but a comparison with the depression of the classical antibody reaction seems at once to suggest itself. A comparison with the results of Craddock and Lawrence (1948) is especially suggestive. These authors irradiated rabbits by a method similar to ours, giving 250 r (measured in air) and studied simultaneously the response to typhoid H and O agglutinogens and to sheep red cells. Our results tally with theirs in three separate respects :

(*a*) If irradiation is given just before the immunizing injection, antibodies do not appear until about twenty days later. Similarly, irradiation on the day before transplantation in our case deferred the destruction of the transplants for a period which appeared to be in the most successful cases of about the same length. The depression of circulating lymphocytes (which is the most readily measurable radiation effect) lasts with this dose of radiation for about the same length of time.

(*b*) If a second dose of the antigen is given after the effects of the radiation have ceased, the secondary response to the booster dose is the same as it would have been if the response to the first dose had not been modified by irradiation. This corresponds exactly to our finding (Fig. 6) that a second set of transplants dies as rapidly after a first set with irradiation as after a non-irradiated first set.

(*c*) Irradiation given after the antigen had been injected did not interfere with the antibody response. In our experiments, once the immune reaction has been established by a first set of homotransplants, irradiation does not prolong the survival of a second set (Fig. 7).

This close correspondence of the two sets of findings we regard as excellent evidence for the basic similarity of the mechanisms involved. It does not afford any positive evidence as to the nature of the immune response. We think it reasonable, however, to draw the same conclusion as Craddock and Lawrence as to the role of the lymphocyte. The simple experiment of irradiation *before* introduction of the antigen, be it bacteria, red cells, or skin, produces a retardation of the immunization corresponding quite closely in time with the fall in circulating lymphocytes, and seems strong evidence that the lymphocyte (or some other cell or structure of a similar grade of radio-sensitivity to the lymphocyte) is the responsible agent. This fits well with the increasing body of evidence for the essential role of the lymphocyte in antibody formation (McMaster and Kidd, 1937 ; Harris, Grimm, Mertens and Ehrich, 1945 ; Dougherty, Chase and White, 1945 ; Harris and Harris, 1948), and might be used equally by those who regard

EXPLANATION OF PLATES.

FIG. 8.—Normal rabbit epidermis and hair follicles (H. & E. × 168). This is recorded as "1½" cells thick.

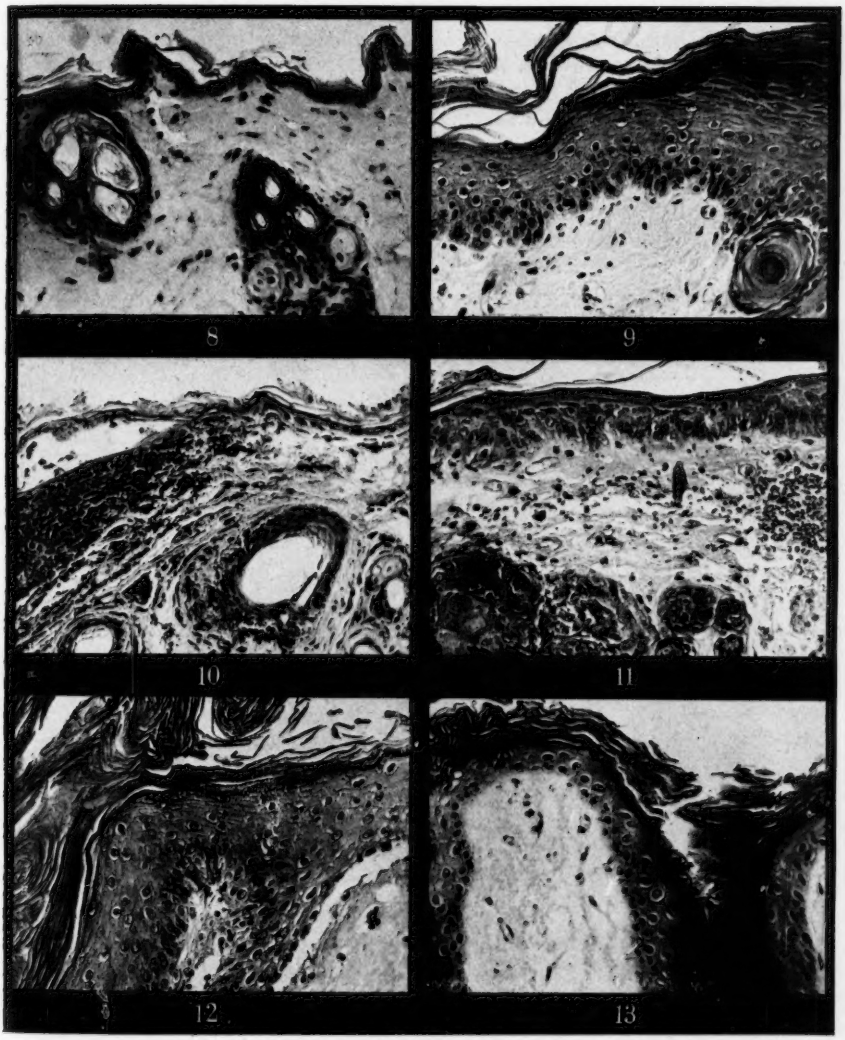
FIG. 9.—Autotransplant, 12th day (H. & E. × 168). Showing the marked hyperplasia normal under these circumstances. Recorded as 10 + cells thick.

FIG. 10.—Homotransplant, 8th day (H. & E. × 168). Epidermis thin and necrotic ; intense cellular infiltration. Recorded as 1 cell thick (necrotic). "Necrotic" is used here in the sense of still present as a tisusue, but judged incapable of survival.

FIG. 11.—Homotransplant with irradiation, 8th day (H. & E. × 168). Active hyperplasia with many mitoses ; moderate cellular infiltration. Recorded as 5 cells thick. (Fig. 11, 12, and 13 are all from our case with longest survival.)

FIG. 12.—Homotransplant with irradiation, 20th day (H. & E. × 168). Stationary phase of maximal hyperplasia. Recorded as 7 cells thick.

FIG. 13.—Homotransplant with irradiation, 24th day (H. & E. × 168). Regression of hyperplasia, as seen at the same stage in autotransplants. Recorded as 5 cells thick, though the area photographed is barely 4. Necrosis occurred within two days of taking this specimen.



Dempster, Lennox and Boag.

the lymphocytic reaction as an essential part of the response to homotransplants (Blumenthal, 1941 ; Loeb, 1944). Satisfactory though it seems, it does not explain the parallel findings of Craddock and Lawrence and ourselves on the effect of irradiation *after* introduction of the antigen. It seems that once the immune reaction is established (or in fact, as the bacterial experiments showed, even merely initiated) irradiation does not interfere with it : the antibody titre continues to rise or the second set of grafts is destroyed, in spite of irradiation enough to maintain a low level of circulating lymphocytes and to damage lymphoid tissue throughout the body.

It appears very much as though two separate mechanisms at least are necessary. One, the receptor, which is necessary for the initiation of the immune response, is highly radiosensitive. The second, the effector, which manufactures the antibody (or in some way contrives the death of homotransplants without manufacturing antibody) is not radiosensitive. One might speculate that the first mechanism resides in the more differentiated elements of the lymphoid apparatus, and that the second in the less differentiated elements which, as Tullis (1949), and Barrow, Tullis and Chambers (1949) have emphasized, are much more resistant. It is clear, however, that the lymphocytes which have so often been ascribed a major role in the death of foreign epithelium or transplanted tumour cannot have any part in the actual destruction of the graft.

SUMMARY.

A dose of 250 r to rabbits given before the application of skin grafts from another rabbit very markedly improves the survival of the grafts, some improvement in all of sixteen cases being obtained and survival of 24 days being produced in one case.

The survival of a second set of grafts from the same donor is not improved by irradiation before the first set or even immediately before the second set.

The close analogies which can be drawn with the effect of irradiation and the development of antibodies against bacteria are believed to be evidence of the immune-reaction nature of the homotransplant reaction.

REFERENCES.

Barrow, J., Tullis, J. L., and Chambers, F. W., jun.—(1949) Naval Medical Research Institute Project NM 007 039, *Report No.* 24.

Bloom, W.—(1948) ' The Histopathology of Irradiation.' New York (McGraw Hill).

Blumenthal, H. T.—(1941) *Arch. Path.*, **31**, 295.

Craddock, C. G., jun., and Lawrence, J. S.—(1948) *J. Immunol.*, **60**, 241.

Dougherty, T. F., Chase, J. H., and White, H.—(1945) *Proc. Soc. exp. Biol. N.Y.*, **58**, 135.

Hagen, C. W., Jacobson, L. W., Murray, R., and Lear, P.—(1949) United States Atomic Energy Commission Reports, MDDC, 999.

Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E.—(1945) *J. exp. Med.*, **81**, 73.

Idem and Harris, Susannah.—(1948) *Proc. Soc. exp. Biol., N.Y.*, **69**, 18.

Hektoen, L.—(1915) *J. infect. Dis.*, **17**, 415.—(1918) *Ibid.*, **20**, 28.—(1920) *Ibid.*, **27**, 23.

Loeb, L.—(1944) 'The Biological Basis of Individuality.' Ill.. (Thomas Springfield).

McMaster, P. D., and Kidd, J. G.—(1937) *J. exp. Med.*, **66**, 73.

Medawar, P. B.—(1944) *J. Anat., Lond.*, **78**, 176.—(1945) *Ibid.*, **79**, 157.—(1946*a*) *Brit. J. exp. Path.*, **27**, 9.—(1946*b*) *Ibid.*, **27**, 15.—(1948*a*) *Ibid.*, **29**, 58.—(1948*b*) *Quart. J. micr. Sci.*, **89**, 239.

Murphy, J. B., and Sturm, E.—(1925) *J. exp. Med.*, **141**, 245.

Tullis, J. L.—(1949) *Amer. J. Path.*, **25**, 829.

THE PROCESS OF CYTOLYSIS IN MOUSE LIVER *IN VITRO*.

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MUCH of the early work on autolysis *in vitro* was done with emulsified tissues, bacterial action being prevented by the addition of antiseptics such as toluene, salicylic and benzoic acids, and after different periods chemical analyses were made to estimate the extent of protein disintegration. With the development of the aseptic technique for the removal of organs from the body, morphological studies were made on tissues removed aseptically and kept under sterile conditions in physiological salt solution for varying periods at different temperatures. Using this method to study autolysis *in vitro*, Wells in 1906, and Launoy in 1909, observed a granular change in the cytoplasm followed by solution and various nuclear changes, namely pyknosis, hyperchromatosis, karyorrhexis and karyolysis. Launoy found that the temperature at which autolytic changes occurred most rapidly was at, or near 37° C., and that at 8–10° C., even after 30 days, the tissues were remarkably well preserved.

Wells believed that the salt solution had a marked solvent effect on the cytoplasm as the solution occurred earliest in the cells at the periphery of the specimens, these cells being in direct contact with the fluid. Cruickshank (1912) was of the same opinion and he compared the changes observed in tissues kept in physiological salt solution with those kept in a moist chamber. He found that in the latter no solution of the cytoplasm occurred unless there was condensation on the surface of the tissue when the superficial cells absorbed the fluid and showed some solution of cytoplasm. On the other hand he observed solution of the cytoplasm in the specimens kept in the physiological saline.

As regards the various nuclear changes that have been described during autolysis *in vitro*, it has not yet been shown whether they represent stages in a single process or whether different conditions are necessary for the production of each type of degeneration.

The present work was undertaken to try to determine whether the cytoplasmic and nuclear changes that take place during autolysis *in vitro* follow a definite pattern and whether the changes in the nuclei are dependent on those in the cytoplasm. In addition an attempt has been made to try to determine the time relationships for the various stages of degeneration under different experimental conditions. It was hoped that these morphological studies on cell death *in vitro* might help to elucidate the spontaneous as well as the radiation induced degenerative changes found in tumours.

MATERIALS AND METHODS.

The livers of newborn mice were cut into small fragments of approximately 2 to 3 mm. in length and width and 1 to 2 mm. in thickness, and kept in various fluids and gas mixtures under sterile conditions for periods varying from 9 to 22 days. One piece was fixed immediately and served as the control. In the first experiment the effect of temperature on the rate of autolysis was examined. Pieces of liver were kept in 200 ml. pyrex conical flasks containing 50 c.c. of unbuffered Ringer solution at room temperature, 7° C. and 37° C. Subsequent experiments were made at room temperature with the same type of flask but the solution in the flask was varied and in some the air replaced by nitrogen which was bubbled through the fluid. The experiments made are shown in Table I.

TABLE I. *Summary of Experiments performed.*

Exp.	Fluid.	pH of fluid.	Gas.	Temperature.
1	Unbuffered Ringer	6·0	Air	37° C.
2	,, ,,	6·0	,,	7° C.
3	,, ,,	6·0	,,	Room
4	,, ,,	6·0	Nitrogen	,,
5	Buffered Ringer	7·4	Air	,,
6	,, ,,	7·4	Nitrogen	,,
7	Potassium cyanide M/1000 in Ringer	7·0	Air	,,
8	Ditto	7·0	Nitrogen	,,
9	Sodium azide M/500 in Ringer	6·5–6·8	Air	,,
10	Ditto	6·5–6·8	Nitrogen	,,
11	Sodium fluoride M/50 in Ringer	7·0	Air	,,
12	Ditto	7·0	Nitrogen	,,
13	Copper sulphate M/5000 in Ringer	6·0	Air	,,
14	Copper sulphate M/10,000 in Ringer	6·0	,,	,,
15	Copper sulphate M/100,000 in Ringer	6·0	,,	,,

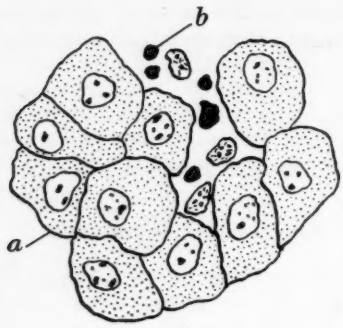
Most of the tissues were fixed in Susa solution, embedded in paraffin and sectioned at 8μ. The sections included the central and peripheral parts of the fragments and were stained with Ehrlich's haematoxylin and counterstained with Biebrich's scarlet. Wilder's stain was used to demonstrate the reticulum and Feulgen's stain for desoxyribose nucleic acid. Some tissues were fixed in 1 in 4 formol-saline and frozen sections were cut and stained for fat with Sudan III.

DEFINITION OF THE AUTOLYTIC PROCESSES.

In describing the cytoplasmic and nuclear changes observed in the various experiments the following terms are used:

Autolytic processes in the cytoplasm.

The initial stage of cytoplasmic autolysis consists of shrinkage of the cell and the appearance of granules in the cytoplasm. Subsequently these may disappear and the cytoplasm fails to stain with Biebrich's scarlet (liquefaction, Fig. 2), or



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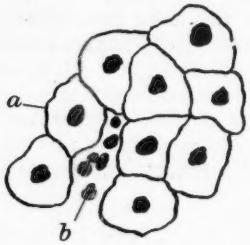

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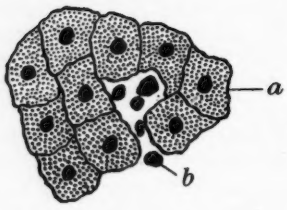

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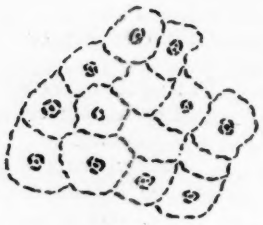

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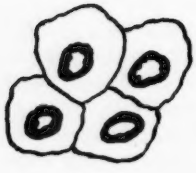

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Camera lucida drawings showing the autolytic changes in the liver cells (*a*) and blood cells (*b*) under the various experimental conditions.

FIG. 1.—Liver cells and blood cells of a newborn mouse, the liver of which was fixed immediately after removal. The nucleus of a primitive blood cell is shown to the right of the one marked (b). Control. × 880.

FIG. 2.—Shrinkage of the liver cells with liquefaction of the cytoplasm and pyknosis of their nuclei. Blood cell nuclei exhibit pyknosis. × 880.

FIG. 3.—Shrinkage of liver cells with condensation of the cytoplasm and pyknosis of their nuclei. Blood cell nuclei also pyknotic. × 880.

FIG. 4.—Liver cells showing karyolysis. × 880.

FIG. 5.—Liver cells illustrating hyperchromatosis of the nuclear membrane. × 880.

FIG. 6.—Nuclei of blood cells showing chromatorrhexis. × 1880.

FIG. 7.—Nuclei of blood cells showing karyorrhexis. × 1880.

the granules may become densely packed and stain a dark pink colour (condensation, Fig. 3). The former change (" liquefaction ") starts around the nucleus and spreads outwards. The final stage of autolysis is termed complete necrosis and it indicates the stage in which there is disappearance of nuclear structures so that only remnants of cellular structure remain.

The rate at which autolysis progresses varies with experimental conditions and may vary for different cells in the same specimen.

Autolytic processes in the nuclei.

At first the chromatic material in the liver cell nuclei becomes aggregated at the nuclear membrane. This is termed hyperchromatosis of the nuclear membrane (Fig. 5). Subsequently the nucleus shrinks and stains homogeneously and intensely : this is pyknosis (Fig. 2 and 3). Later the nuclei stain faintly indicating karyolysis (Fig. 4) and finally they fail to react to Feulgen or haematoxylin staining (complete necrosis). In the same specimen nuclei of adjacent cells may be in different stages of autolysis at one time.

The liver of newborn mice contains lymphocytes with small densely staining nuclei and primitive blood cells with larger and paler nuclei. The first stage of autolysis in these blood cells is shrinkage of the lymphocytes and pyknosis of the more primitive stem cells. This is followed by chromatorrhexis, i.e. fragmentation of the chromatic material within an intact nuclear membrane. This process involves the separation of chromatic from nonchromatic material within the nucleus and thus the appearance of pale staining regions (Fig. 6). In the next stage termed karyorrhexis the nuclear membrane is no longer distinguishable (Fig. 7). The final stage is complete necrosis when the granules fail to stain with nuclear stains.

Both types of these blood cells contain very little cytoplasm which disappears early on in the autolytic process.

EXPERIMENTAL RESULTS.

Experiments 1 to 3 (unbuffered Ringer in air at 37° C., 7° C. and at room temperature) were undertaken to study the variation in the rate of autolysis with temperature. At the higher temperatures the rate of autolysis was more rapid and the process more complete.

In all three experiments the cytoplasm of the liver cells was at first granular and subsequently showed liquefaction.

At 37° C. the liver cell nuclei in the central part of the fragment proceeded rapidly through the stages of hyperchromatosis of the nuclear membrane, pyknosis and karyolysis to complete necrosis which was reached in 3 days, while the nuclei of the liver cells in the peripheral part of the fragment did not show complete necrosis until 10 days.

At room temperature autolysis of the liver cell nuclei proceeded more slowly but, as at 37° C., autolysis progressed more rapidly in the central part of the fragment than in the peripheral part. In the central part the liver cell nuclei reached the stage of complete necrosis in 10 days while in the peripheral part the nuclei were still in the stage of karyolysis after 14 days.

At 7° C. the rate of progress of autolysis was relatively slow, the liver cell nuclei

only reached the stage of pyknosis in 10 days, and the rate was the same throughout the fragment until the 14th day when the liver cell nuclei in the central part of the fragment reached the stage of karyolysis while those in the peripheral part remained pyknotic.

At 37° C. the autolytic change in the blood cell nuclei proceeded via pyknosis, chromatorrhexis and karyorrhexis to complete necrosis which was reached in 3 days in the central part of the fragment but not until 10 days in the peripheral part.

At room temperature the process was slower and in the central part of the fragment the stage of complete necrosis was only reached after 10 days while in the peripheral part the nuclei of the blood cells were still in the stage of karyorrhexis after 14 days.

At 7° C. the rate of progress of autolysis was even slower and the stage of complete necrosis was not reached even after 14 days. The nuclei of the blood cells in the central part of the fragment reached the stage of karyorrhexis in 13 days while those in the peripheral part only reached the stage of chromatorrhexis.

In these experiments the fat content of the liver cells undergoing autolysis was studied. In the early stages of autolysis the amount of stainable fat increased slightly but then remained more or less constant. In one experiment no fat was present in the control specimen and none appeared as autolysis progressed.

Experiments 1 to 3 show that during autolysis there is " unmasking " of the fat in the liver cells but no real increase in the amount of fat. They also show that the rate of autolysis varies with temperature and that it is more rapid in the central parts of the fragments than in the peripheral parts.

Experiments 4 to 6 were undertaken to study (*a*) the effect of oxygen concentration on the rate of autolysis in the central and peripheral parts of the liver fragments, and (*b*) the effect of acidity on the autolytic process.

In Experiment 4 (unbuffered Ringer in nitrogen at room temperature) the cytoplasm of the liver cells appeared at first granular and subsequently condensed. The rate of progress of autolysis in the liver fragment was the same in the central and peripheral parts up to 16 days, during which time the liver cell nuclei proceeded via hyperchromatosis of the nuclear membrane and pyknosis to karyolysis, and the nuclei of the blood cells via pyknosis and chromatorrhexis to karyorrhexis. After 16 days the nuclei of the liver cells and blood cells in the central part of the fragment reached the stage of complete necrosis while those at the periphery remained in the stages of karyolysis and karyorrhexis respectively. Thus the nitrogen slowed down degeneration but did not affect the difference in the rate of autolysis in the central and peripheral parts of the fragments.

In Experiment 5 (buffered Ringer in air at room temperature) and Experiment 6 (buffered Ringer in nitrogen at room temperature) the rate of progress of autolysis was the same in the central and peripheral parts of the liver fragments, but the whole process was retarded as compared with Experiments 3 and 4 since even after 20 days the autolytic process had not reached the stage of complete necrosis.

In Experiment 5 the cytoplasm of the liver cells was at first granular and subsequently showed liquefaction while in Experiment 6 the initial granular change was followed by condensation. In these two experiments the nuclei of the liver cells passed through the stage of hyperchromatosis of the nuclear

membrane, pyknosis and karyolysis which was first evident after 12 days and was still present after 20 days. The nuclei of the blood cells proceeded via pyknosis and chromatorrhexis to karyorrhexis which was reached in 12 days and was still present after 20 days.

Experiments 4 to 6 show that lack of oxygen retards the rate of autolysis but does not affect the difference in rate between the centre and periphery, while reduction of acidity eliminates the difference. These experiments also show that in the fragments kept in nitrogen (Experiments 4 and 6) the cytoplasm of the liver cells was condensed while in those kept in air (Experiment 5) it was liquefied.

In order to investigate further the role played by oxygen in autolysis some experiments with enzymic inhibitors were undertaken and cyanide, azide and fluoride were chosen for that purpose. Cyanide is known to inhibit a great many metaloprotein enzymes including cytochrome oxidase which is involved in intracellular respiration. In addition it has an enhancing effect on the activity of certain hydrolytic enzymes, notably some proteolytic ones. Azide, like cyanide, is a strong inhibitor of the metaloprotein enzymes but it has no enhancing effect on the proteolytic ones. Fluoride is known to inhibit several phosphate-transferring enzymes involved in the normal anaerobic carbohydrate metabolism of animal tissues and those that require magnesium as coenzyme are particularly sensitive to the action of fluoride (cf. Sumner and Somers, 1943).

With cyanide there was no difference in the rate of autolysis in the fragment kept in air (Experiment 7, potassium cyanide in unbuffered Ringer in air at room temperature) and in that kept in nitrogen (Experiment 8, potassium cyanide in nitrogen at room temperature). As compared with the previous experiments the rate of progress of autolysis was similar to that in Experiment 3 (unbuffered Ringer in air at room temperature) and, as in Experiment 3, autolysis progressed more rapidly in the central part of the fragment than in the peripheral part.

In Experiment 7 the cytoplasm was at first granular and subsequently became liquefied, while in Experiment 8 the initial granular change was followed by condensation. In both experiments the liver cell nuclei passed through the stages of hyperchromatosis of the nuclear membrane, pyknosis and karyolysis and in the central parts of the fragments complete necrosis occurred after 9 days while at the preiphery the liver cell nuclei remained in the stage of karyolysis. The stages of autolysis in the blood cell nuclei were pyknosis, chromatorrhexis and karyorrhexis and in the centre of the fragments the nuclei showed complete necrosis at 9 days while in the periphery they remained in the stage of karyorrhexis.

Thus cyanide does not retard autolysis nor does it affect the difference in rate between centre and periphery. As regards cytoplasmic autolysis cyanide does not vitiate the difference due to nitrogen and oxygen in the atmosphere.

The effect of sodium azide was to slow the rate of autolysis as even after 21 days the stage of complete necrosis was not reached. In Experiment 9 (sodium azide in unbuffered Ringer in air at room temperature) the cytoplasm of the liver cells was granular for 6 days and then liquefaction commenced around the nucleus but it did not progress further than this stage of perinuclear liquefaction. The liver cell nuclei passed through the stages of hyperchromatosis of the nuclear membrane, pyknosis and karyolysis, which was reached after 16 days in the central part of the fragment but not until 19 days in the peripheral part. In the

central part the blood cell nuclei proceeded via pyknosis and chromatorrhexis to karyorrhexis which was reached in 16 days while at the periphery they only reached the stage of chromatorrhexis.

In Experiment 10 (sodium azide in unbuffered Ringer in nitrogen at room temperature) the rate of autolysis was retarded as in Experiment 9 but it was the same throughout the whole fragment. The cytoplasm of the liver cells was at first granular and after 3 days it was condensed. The nuclei of the liver cells proceeded through the stage of hyperchromatosis of the nuclear membrane to pyknosis which was reached in 6 days and they remained in this stage until the 21st day. The nuclei of the blood cells proceeded via pyknosis to chromatorrhexis which was reached in 16 days and they remained in this stage for 5 days.

Thus azide retards the rate of autolysis more markedly in nitrogen than in air, and while there is a difference in the rate of autolysis in centre and periphery in the fragments kept in air, the slow progress of autolysis obscures any difference between centre and periphery in the fragments kept in nitrogen. As regards cytoplasmic autolysis azide does not vitiate the difference due to nitrogen and oxygen in the atmosphere.

In Experiment 11 (fluoride in unbuffered Ringer in air at room temperature) the cytoplasm was granular for 6 days and then showed perinuclear liquefaction which persisted for 21 days. The rate of autolysis was retarded to the same extent as with azide in unbuffered Ringer in air but it was the same throughout the fragment. The nuclei of the liver cells passed through the stages of hyperchromatosis of the nuclear membrane, pyknosis and karyolysis which was reached in 16 days and even after 21 days complete necrosis had not occurred. The nuclei of the blood cells passed through the stages of pyknosis, chromatorrhexis and karyorrhexis which was reached in 19 days and the stage of complete necrosis was not reached even at 21 days.

In Experiment 12 (fluoride in unbuffered Ringer in nitrogen at room temperature) autolysis progressed very rapidly and the whole fragment with the exception of a very narrow peripheral rim, in which there was still some nuclear staining, reached the stage of complete necrosis in 3 days. The cytoplasm of the liver cells was at first granular and after 3 days was condensed. The nuclei of the liver cells passed through the stages of hyperchromatosis of the nuclear membrane, pyknosis and karyolysis to complete necrosis on the 3rd day and the nuclei of the blood cells proceeded via pyknosis to complete necrosis in 3 days.

Thus fluoride in air retards the rate of autolysis and affects the differential rate between centre and periphery. Fluoride in nitrogen accelerates the rate of autolysis and does not alter the differential rate between centre and periphery. As regards cytoplasmic autolysis fluoride does not vitiate the difference due to nitrogen and oxygen in the atmosphere.

Experiments 13 to 15 were undertaken to study the effect of inhibiting proteolytic enzymes by means of copper sulphate in various dilutions. In all three experiments, however, the copper sulphate acted as a fixative and even after 20 days the tissues were as well preserved as the controls which were fixed immediately after removal from the mouse. The cytoplasm of the liver cells showed no autolytic changes and both the liver cell and blood cell nuclei retained their normal structure.

*Size of cells and nuclei during autolysis.**

The observed liquefaction of the cytoplasm of the liver cells in Experiments 1, 2, 3, 5, 7, 9 and 11 might be explained on the basis of swelling while the condensation observed in Experiments 4, 6, 8, 10 and 12 might be due to shrinkage of the cells and for that reason it was thought worth while to measure the diameter of the cells in representative areas of the sections using a micrometer eyepiece with oil immersion objective. Measurements were made on the control sections and on the 4th and 8th days in the fragments kept in unbuffered Ringer in air and in nitrogen and in those kept in buffered Ringer in air and in nitrogen. Two hundred cells or nuclei were measured in each section, the nuclei in one diameter and the liver cells in two diameters. In the latter the mean of the two diameters was taken as the value indicating the size of the cell.

The liver cells shrunk more rapidly in nitrogen than in air whether the fragments were kept in buffered or unbuffered Ringer. In addition the liver cell size decreased more rapidly in unbuffered than in buffered Ringer whether the fragments were kept in air or in nitrogen. With the fragments kept in unbuffered Ringer in nitrogen the rate of shrinkage was very rapid during the first 4 days and reduced the mean diameter from 14μ to 8·5μ whereas in buffered Ringer in nitrogen the mean diameter was 10·1μ. In the fragments kept in air during the first 4 days the mean diameter was reduced to 11·0μ in unbuffered Ringer and 11·7μ in buffered Ringer.

If autolysis is rapid the cells in the centre of the fragment shrink more rapidly than those at the periphery, thus in the fragments kept in unbuffered Ringer in air on the 8th day the mean diameter of the liver cells was 10μ at the periphery and 8·5μ in the centre.

In the fragments kept in air the cells looked as though they were swollen while in the fragments kept in nitrogen the cells looked dense and compact. Measurements showed that some of this difference might be accounted for by the more rapid shrinkage of the liver cells in nitrogen, but prove that the cells of the fragments kept in air have not swollen but on the contrary have shrunk.

The measurements of the nuclei of the liver cells and blood cells showed no difference in the rate of shrinkage with lack of oxygen or buffering of the Ringer. The mean diameter of the liver cell nuclei fell from about 7·1μ–3·5μ during the first 8 days while that of the blood cell nuclei fell from about 4·7μ–2·5μ. The behaviour of the nuclei contrasts with that of the cytoplasm under the conditions of these experiments.

DISCUSSION.

From a study of the morphological changes seen in the various experiments it appears that the cytoplasmic and nuclear changes that take place during autolysis do so independently of each other since the same nuclear changes occur whether the cytoplasm shows liquefaction or condensation. This is well illustrated in the experiments with buffered Ringer: in spite of the marked difference in the cytoplasmic changes in air and in nitrogen the changes in the nuclei of the liver cells were the same in the two experiments.

In trying to trace a pattern for the nuclear changes during autolysis the follow-

* Tables giving the details of the measurements of the liver cells and their nuclei and of the nuclei of the blood cells, and details of the duration of the stages of autolysis have been deposited with the Librarian, General Library, British Museum (Natural History), London, S.W.7.

ing facts emerge : pyknosis may end in karyolysis or karyorrhexis and there is nothing obvious in the appearance of the pyknotic nucleus to indicate which change will ensue. If however, pyknosis is preceeded by hyperchromatosis of the nuclear membrane karyolysis will follow, and if not chromatorrhexis and karyorrhexis will ensue.

Under the various experimental conditions described the pattern of the nuclear changes remained constant though the rate of change was affected by experimental conditions. Where the duration of autolysis was increased it was so equally for all the stages of the process.

Experiments (1 to 3) undertaken to study the effect of temperature on autolysis confirmed the results of previous investigators (Launoy, 1909) that autolysis occurs most rapidly at 37° C. and that temperatures below this have an inhibitory effect on the rate of the autolytic process.

Whether the increase in the fat content of cells during autolysis is real or merely apparent has been the subject of much controversy. Cruickshank (1912) investigated this point in his studies on autolysis in the organs of guinea-pigs and did not find any fat. In one experiment of the present work no fat was demonstrated either in the control or during autolysis, and in other experiments after a slight initial increase in the amount of stainable fat in the cells the quantity remained more or less constant. Both these observations seem to confirm the view that during autolysis there is no new formation of fat but that the fat present in the cells is " unmasked " and so becomes stainable.

The cytoplasm may undergo either liquefaction or condensation. Cruickshank (1912) and Wells (1906) noted solution of the cytoplasm, first evident at the periphery of the specimens, in their investigations on autolysis *in vitro*, and they believed it was due to the solvent action of the salt solution in which the tissues were kept. In the present work all the specimens were kept in a salt solution, but liquefaction did not always occur. When it did it extended throughout the fragments, and was not first obvious at the periphery of the specimens.

In the description of the experimental results the degree of liquefaction was not stressed but it varied in the different experiments. It was very rapid and complete with buffered Ringer, less rapid but ultimately complete with unbuffered Ringer and with cyanide, whilst with azide and fluoride it did not progress further than the initial stage of perinuclear liquefaction. The same nuclear change, e.g. pyknosis, occurred with various degrees of liquefaction and this illustrates again that the cytoplasmic and nuclear changes are not closely related to each other.

Liquefaction did not take place whenever the fragments were kept in nitrogen but always did so when kept in air whatever the salt solution used. On the other hand anaerobic conditions by themselves do not seem to account for the difference in cytoplasmic autolysis since in the experiments with cyanide and azide, both of which should cause anaerobic conditions by their inhibition of cytochrome oxidase, the different cytoplasmic changes still occurred according to whether the fragments were kept in air or in nitrogen.

The degenerative changes observed under low atmospheric and oxygen pressures seem to be different from those observed under anaerobic conditions in the present work. In 1942 Pichotka reported some experiments on guinea-pigs exposed in chambers to low atmospheric and oxygen pressures and found that after a few minutes to a few hours the liver cells showed a vacuolar degeneration. The vacuolation was not due to fat and Pichotka was of the opinion that it might

be due to glycogen or to some type of protein deposit. In 1942 Hesse reported a similar change in the liver cells following death due to acute oxygen lack, as did also Müller and Rotter (1942) in their study of post-mortem material of people who had died in an air crash. In the present investigation this vacuolar degeneration was not found in any of the experiments made under anaerobic conditions.

The mechanism by which nitrogen produces condensation of the cytoplasm instead of liquefaction is obscure. Nitrogen was originally tried in order to test whether the more rapid autolysis in the centre as compared with the periphery of the fragments could be explained by lack of oxygen. The experiments show that there is no such correlation.

More rapid degenerative changes in the central parts of liver transplants have been described by Duthie (1935). The work of Morse (1916), Bradley (1915) and Bradley and Taylor (1916) showed that the rate of autolysis in liver pulp varied with the pH of the substrate. Maximum autolysis occurred at about pH 6·0 while at pH 7·4–7·8 it was reduced to a minimum. This suggested that changes in pH rather than changes in oxygen tension might be responsible for the different rate of autolysis in the centre and periphery of the fragments. The experiments showed that when the fragments were kept in a buffered Ringer solution of pH 7·4 the whole autolytic process was slowed and any difference in the rate of autolysis at centre and periphery prevented.

The experiments with enzymic inhibitors are difficult to interpret. As cyanide is an inhibitor of cellular respiration it may be expected to exclude the part normally played by oxygen and create anaerobic conditions. In other words, one would expect that the autolytic processes, if at all influenced by the presence of oxygen as such, might be similar in nitrogen and in air so long as cyanide is present. This was in fact true of the nuclear changes but not of the cytoplasmic ones which still differed in nitrogen and in air.

Autolysis occurs more slowly under anaerobic conditions and thus with cyanide a slow rate of autolysis might have been expected. This was not the case as the rate equalled that of the fragments kept in unbuffered Ringer in air. Since cyanide has an enhancing effect on some proteolytic enzymes it is possible that the inhibition ot autolysis by anaerobic conditions may have been balanced by the stimulation of proteolytic enzymes. It is interesting in this connection that sodium azide which is an inhibitor of intracellular oxidative processes but not a stimulator of proteolytic enzymes had a marked inhibiting effect on the rate of autolysis.

In the experiments with fluoride autolysis was very rapid in the fragments kept in nitrogen whereas in those kept in air the rate of autolysis was slowed to the same extent as with azide. Although principally concerned with phosphorylations, fluoride may have a different mode of action under aerobic and anaerobic conditions.

It is interesting to compare the time relationships of cellular degenerations that occur *in vitro* with those *in vivo*. Mönnighoff (1939) showed that autolysis occurred more rapidly if dead mice were kept whole than if the organs were removed from the body and kept separately under the same conditions. *In vivo* cellular degenerations occur more rapidly than *in vitro*. Cellular degenerations occur during normal development, and in 1940 Glücksmann showed that during normal development of the frog tadpole retina the process of degeneration took about 7 hours. In 1938 Spear and Glücksmann found that in tadpoles exposed

to radium the whole process took between 1 and 3 hours. In experiments on tissue cultures Strangeways and Oakley (1923) noted that the process of cellular degeneration could be completed in ½ to 1 hour, and Fell and Andrews (1927) in observations on tissue cultures of Jensen's rat sarcoma found that in some cells the degenerative process took about 7 hours. In the present work the duration of the autolytic process varied from 3 days to 21 days or more depending on various experimental conditions.

While the rate and process of cytoplasmic autolysis varied under experimental conditions, the nuclear changes have been remarkably constant in spite of the altered cytoplasmic changes. There appear to be two distinct mechanisms of nuclear disintegration, the one leading via hyperchromatosis of the nuclear membrane to pyknosis and karyolysis and the other via pyknosis to chromatorrhexis and karyorrhexis.

SUMMARY.

1. The cytoplasmic and nuclear degenerative changes that occur during autolysis in mouse liver under varying experimental conditions are described.

2. The cytoplasmic changes are of two types—liquefaction and condensation—which are correlated with the presence of air or nitrogen in the atmosphere.

3. The nuclear changes are independent of the cytoplasmic ones and conform to a definite pattern.

4. The rate of autolysis varies with temperature.

5. The rate of autolysis varies in the centre and periphery of the liver fragments and is correlated with the acidity of the medium.

6. The question of the production of fat during autolysis is discussed and the view that the fat in the cell is " unmasked " is confirmed.

7. The effect of some enzymic inhibitors (cyanide, azide, fluoride and copper sulphate) on autolysis is discussed.

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REFERENCES.

Bradley, H. C.—(1915) *J. biol. Chem.*, **22**, 113.
Idem and Taylor, J.—(1916) *Ibid.*, **25**, 261.
Cruickshank, J.—(1912) *J. Path. Bact.*, **16**, 167.
Duthie, E. S.—(1935) *Ibid.*, **41**, 311.
Fell, Honor B., and Andrews, J. A.—(1927) *Brit. J. exp. Path.*, **8**, 413.
Glücksmann, A.—(1940) *Brit. J. Ophthal.*, **24**, 153.
Hesse, W.—(1942) *Beitr. path. Anat.*, **107**, 173.
Launoy, L.—(1909) *Ann. Inst. Pasteur.*, **23**, 1.
Mönnighoff, F. H.—(1939) *Beitr. path. Anat.*, **102**, 87.
Morse, M.—(1916) *J. biol. Chem.*, **24**, 163.
Müller, E., and Rotter, W.—(1942) *Beitr. path. Anat.*, **107**, 156.
Pichotka, J.—(1942) *Ibid.*, **107**, 117.
Spear, F. G., and Glücksmann, A.—(1938) *Brit. J. Radiol.*, **11**, 533.
Strangeways, T. S. P., and Oakley, H. E. H.—(1923) *Proc. roy. Soc.*, B, **95**, 373.
Sumner, J. B., and Somers, G. F.—(1943) 'Chemistry and Methods of Enzymes.' New York, N.Y. (Academic Press Inc.).
Wells, H. G.—(1906) *J. med. Res.*, **15**, 149.